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Research paper

Waterlogging and soil freezing during dormancy affected root and shoot phenology and growth of Scots pine saplings

Marja Roitto[1], Sirkka Sutinen[1], Ai-fang Wang[1,2], Timo Domisch[1], Tarja Lehto[3] and Tapani Repo[1,4]

[1]Natural Resources Institute Finland (Luke), Natural Resources, Joensuu, PO Box 68, FI-80100 Joensuu, Finland; [2]Present address: College of Horticulture, Agricultural University of Hebei, 071001 Baoding, China; [3]School of Forest Sciences, University of Eastern Finland, PO Box 111, FI-80101 Joensuu, Finland; [4]Corresponding author (tapani.repo@luke.fi)

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Soil waterlogging is predicted to increase in the future climate in boreal regions due to increased precipitation. Snowmelt periods in winter may also become more common and further increase the amount of water in soil. It is not well known how waterlogging and soil freezing during winter affect the physiology, phenology and growth of trees. Our aim was to study the below- and aboveground responses of Scots pine (*Pinus sylvestris* L.) saplings to waterlogging (WL) in frozen (Fr) and unfrozen (NoFr) soils in a growth chamber experiment. The soil was either −2 °C or +2 °C and either waterlogged or not in a split-plot design for 6 weeks during dormancy, with similar air conditions in all treatments, which were Fr + WL, NoFr + WL, Fr + NoWL and NoFr + NoWL. Needles showed a shift towards a deeper dormancy in frozen than unfrozen soil in terms of chlorophyll fluorescence (F_v/F_m), water potential and apoplastic electrical resistance. In spring, initiation of shoot elongation started earlier if the soil was frozen during dormancy. In Fr + WL, initiation of root growth was delayed by 20 days compared with other treatments; after that, the root growth peaked at the same time as needle elongation. Needles remained smaller in Fr + WL than in the other treatments, indicating that roots formed a strong sink for carbon. Shoot and root biomass were not negatively affected by waterlogging if the soil remained unfrozen. In Fr + WL, survival and growth capacity of new terminal and whorl buds, the number of bud scales and the number of dwarf shoots were reduced. We conclude that soil freezing on sites prone to waterlogging should be considered in management of boreal forests, especially in the face of predicted climate change.

Keywords: dormancy, growth, phenology, roots, soil freezing, waterlogging.

Introduction

The Scots pine (*Pinus sylvestris* L.) is widely distributed in the boreal and temperate zones, and as a versatile conifer it is capable of growing both in dry and wet environments (Mirov 1967, Pearson et al. 2013). Different genotypes are adapted to a large seasonal and annual variation in climate conditions. The right timing of phenology and underlying physiological processes of above- and belowground organs are therefore key measures to adaptation on a site, thus affecting carbon allocation as well. Drivers for root development are much less known than those of shoots. Even though roots and shoots are partly autonomous organs, they are interrelated depending on the annual phase of development (Lyr and Hoffman 1967, Radville et al. 2016*a*, 2016*b*). The key drivers for spring and autumn phenophases of aboveground parts are temperature, quality and quantity of light, and photoperiod (Koski and Sievänen 1985, Sutinen et al. 2012, Viherä-Aarnio et al. 2014, Hänninen 2016). Root growth is known to be affected by soil temperature, soil moisture, nutrient availability (extrinsic drivers) and carbohydrate allocation (intrinsic driver), but root phenology and its relation to shoot phenology is not well known (Lyr and Hoffman 1967, Radville et al. 2016*a*, *2016b*).

Winter conditions are predicted to change in the boreal zone due to climate change. Increased precipitation concomitant with reduced snow cover may increase incidences of soil freeze–thaw cycles and increase the depth of soil frost (IPCC 2013), thus increasing the risks of root and shoot damage (Groffman

et al. 2001, Repo et al. 2014). In a Norway spruce stand, increased soil freezing, due the lack of protective snow layer, led to the increase of median longevity of short and long roots by 23 days and 32 days, respectively (Repo et al. 2014). Soil freezing after snow removal increased root mortality in a northern hardwood forest (New Hampshire, USA) (Groffman et al. 2001, Tierney et al. 2001, Cleavitt et al. 2008) and in a Norway spruce stand (SE Germany) (Gaul et al. 2008). These results suggest that soil conditions and their changes in winter impact belowground carbon pools and fluxes in forest ecosystems. However, the effects of soil freezing, together with an elevated water table in winter, on root and shoot phenology and growth of boreal conifers have not been greatly studied (Sutinen et al. 2015).

The boreal forest zone has wide peatland areas with a high water table and large seasonal variation. In those areas, water table is among the most important factors affecting forest regeneration and early development of Scots pine seedlings (Pearson et al. 2011). It is not well known how waterlogging, occurring together with soil freezing in winter, affects the phenology and growth of the Scots pine. During the growing season, the effects may vary from lethal to reversible physiological changes depending on the age of the tree and on the duration of waterlogging (Repo et al. 2016, 2017). During dormancy, the risks due to waterlogging have only been considered to be lower than during the growing season, probably due to lower physiological activity and the oxygen demand of roots (Crawford 2003). Consistent with that, the roots of dormant *Picea sitchensis* and *Pinus contorta* cuttings tolerated 28 days of WL at 6 °C in a laboratory experiment (Coutts and Philipson 1978). Similarly, *Pinus taeda* seedlings tolerated 5 months of waterlogging during dormancy, whereas continuous waterlogging during the growing season decreased both the shoot and root biomass (DeBell et al. 1984). Four weeks of exposure to waterlogging at 2 °C during dormancy did not seriously affect 1-year old *Picea abies* seedlings during the follow-up growing season (Wang et al. 2013), but in that experiment, the soil was not frozen during waterlogging treatment.

Waterlogging brings an additional element to the soil freezing process by increasing mechanical and hypoxic stress on roots. If waterlogging continues from autumn to winter, or if it is created by snowmelt, there may be occasional freezing of water-saturated soil, which may impact trees differently compared with waterlogging of unfrozen soil. Sub-zero soil temperatures as such may damage unhardened roots. Although cold-hardened fine roots of Scots pines tolerate temperatures even below −20 °C (Sutinen et al. 1998, Bigras et al. 2001, Di et al. 2018), soil freezing in winter may damage roots due to frost heaving and/or cellular dehydration, thereby affecting growth during the following growing seasons (Sutinen et al. 1996, 2014, Gaul et al. 2008).

We aimed to study, in a growth chamber experiment, how waterlogging in frozen or nonfrozen soils during dormancy affects the roots and shoots of the Scots pine saplings. We hypothesized that the freezing of waterlogged soil is a harmful condition for roots, thus affecting growth of the saplings during the following growing season.

Materials and methods

Experimental set-up

Sixteen 5-year-old Scots pine (*Pinus sylvestris* L.) saplings were lifted from a forest plantation site in Eastern Finland (62°46′N, 30°08′E, 100 m above sea level) in May 2009. The stand was established in 2005 with 1-year-old nursery seedlings (seed origin SV165 Jörkki) that were planted in moulds. The growth site was paludified *Myrtillus* type. The mean height of the selected saplings was 103 cm, and the trunk diameter at 15 cm above the root collar was 2.2 cm. The saplings were transported into the Joensuu dasotrons and replanted in four root containers in each of the four dasotrons (RTR48, Conviron, Winnipeg, MB, Canada) (Finer et al. 2001).

The volume of the root containers was 0.19 m^3 and each container was equipped with two glycol circulation coils (one at the top and one at the bottom) to control soil temperature separately from air temperature. An insulation sheet was set on the upper cooling element to maintain stable soil temperature and to decrease direct evaporation from the soil. At the bottom of the containers, a 20-cm-thick layer of fine textured sand was set. The saplings were planted in a 30-cm-deep layer of mineral soil (pH 5.3) from the stand where the saplings were lifted. The

Table 1. Soil and air conditions in different phases of the dasotron experiment. Time indicates the end of each period in running days from the beginning of the experiment. 'Trt' indicates the time for cessation of waterlogging and soil frost treatments during dormancy. The growing seasons (G1, G2) are divided into long-day (LD) and short-day (SD) periods. (d/n) refers to day/night and RH to relative humidity.

	Growing season (G1)		Dormancy (D)			Growing season (G2)	
	LD	SD	Pre-Trt	Trt	Post-Trt	LD	SD
Time, days	63	84	105	147	175	238	259
Photon flux density, μmol m^{-2} s^{-1}	300	200	200	200	200	300	200
Photoperiod (d/n), h	18/6	6/18	6/18	6/18	6/18	18/6	6/18
Air temp. (d/n), °C	20/15	20/15	4/2	4/2	4/2	20/15	20/15
Air RH (d/n), %	70/80	70/80	90	90	90	70/80	70/80
Soil temp., °C	15	15	2	2/−2	2	15	15

texture of the mineral soil (sieved by 10 mm grid) was 4% silt (0.002–0.02 mm), 16% fine sand (0.02–0.2 mm), 59% medium sand (0.2–0.6 mm), 16% coarse sand (0.6–2 mm) and 3% gravel (>2 mm). Five-cm-thick soil discs were taken from the topmost organic layer (pH 3.5) from the close to the forest plantation stand (*Myrtillus* type) from where the test saplings were lifted. The organic soil discs were put on the surface of the mineral soil layer of the containers.

After planting, the saplings had a 12-week growing season (G1) (Days 1–84) to become established in the chamber conditions (Table 1). The G1 included a 9-week long-day and a 3-week short-day period. The shift from the short-day phase to the 13-week long dormancy (D) took place in 3 weeks (Days 85–105). Waterlogging and soil frost treatments during the D period lasted for 6 weeks (Days 106–147), followed by a 4-week post-treatment period (Days 148–175). In that period, soil thawed, excess water drained and soil returned to normoxic condition. The follow-up growing season (G2) included a 9-week long-day (Days 176–238) and a 3-week short-day period (Days 239–259) before final harvest.

The experiment had two soil temperature treatments and two soil waterlogging treatments in a split-plot design with two replicates for the temperature and four for the waterlogging treatments, respectively. The soil temperature treatments were soil frozen (Fr) and not frozen (NoFr) and the waterlogging treatments were waterlogged (WL) and not waterlogged (NoWL). Thus, the treatment combinations were Fr + WL, NoFr + WL, Fr + NoWL and NoFr + NoWL. Soil frost treatments (main plots) were randomly assigned to the chambers, and waterlogging treatments (subplots) were randomly assigned among the four pots within each chamber. Thus, each chamber had two WL and two NoWL containers. Waterlogging with lake water (pH adjusted to 5.5) took place by raising the water table to the top level of the organic soil layer (Day 106) before soil freezing was initiated in the Fr treatment. After 6 weeks of WL (Day 147), the valves at the bottom of the root containers were opened for drainage, and soil thawing was started in the Fr treatment. During growing seasons, the soil moisture in the mineral layer was maintained at field capacity (~25%) by irrigation twice a week. The chemical composition of irrigation water was adjusted to correspond to the precipitation in southern Finland (Sallantaus 1992, for ionic composition see Wang et al. 2013).

Soil temperature (105 T thermocouple, Campbell Scientific, Shepshed, UK) and volumetric water content (Theta Probe, ML 2x, Delta-T Devices, Cambridge, UK, and CS615, Campbell Scientific) were measured in the organic layer (2.5 cm from the surface) and in the mineral layer (15 cm from the surface) at 20-min intervals. The air-saturated soil oxygen content was measured weekly in the mineral layer at a depth of 15 cm from the surface by 4-Channel Fibre-Optic Oxygen Meter (OXY-4, PreSens, Germany) using optical sensors (Oxygen Dipping Probe, DP-PSt3-L2.5-St10-Yop, PreSens, Regensburg, Germany).

Measurements of roots

Root growth was followed by means of minirhizotron imaging (Bartz BTC-100X Camera System, Bartz Technology Company, Santa Barbara, CA, USA) four times during the first growing season, two times during dormancy, and six times during the follow-up growing season. The imaging tube (exterior diameter 60 mm) was set horizontally in the mineral layer at the depth of 15 cm from the soil 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 × 18 mm). Short root (first order) and long root (higher than first order) elongation and formation of new root tips were measured from the images by means of the RootView software (Aphalo and Simonic 1999).

In the analysis of the minirhizotron data, the length of roots and the number of root tips of all frames in a tube were aggregated by sampling times. The large variation between the tubes in the root length and in the number of root tips (data vectors) of each tube was considered by equalization of the tubes using Euclidian norm as follows:

$$X_j = \left\{ \frac{x_{1j}}{\sqrt{x_{1j}^2 + x_{2j}^2 + \ldots + x_{nj}^2}}, \frac{x_{2j}}{\sqrt{x_{1j}^2 + x_{2j}^2 + \ldots + x_{nj}^2}}, \ldots, \frac{x_{nj}}{\sqrt{x_{1j}^2 + x_{2j}^2 + \ldots + x_{nj}^2}} \right\}, \quad (1)$$

where X_j is the jth vector (root length and number of tips) of the set of N vectors (i.e., the number of measured tubes) at the sampling times $t = \{t_1, t_2, \ldots, t_n\}$, thereby forming a group of normalized vectors $X = \{X_1(t), X_2(t), \ldots, X_N(t)\}$. By normalization, small changes in the shape of the curves in a particular period became visible. At each time, a normalized number of root tips was calculated. Production of short and long roots was calculated as the difference between the subsequent normalized lengths.

The proportion of dead fine roots out of the total was assessed from the minirhizotron images, and separately for short and long roots throughout the experiment. A root was defined as dead when it started to appear disintegrated in the image. The proportion of dead roots was calculated for each imaging time.

In the final harvest, root biomass was assessed from a sector sample (area 385 cm^2) of each container from organic and three 10-cm-thick mineral layers. Roots (<2.0 mm up to 4.5 mm) were separated from soil and dried for 4 days at 60 °C and measured for dry mass.

Measurements of shoots and needles

Elongation of the leader shoot and elongation of needles were measured with a ruler and the trunk diameter at 15 cm above root collar with a slide gauge one or two times a week in both growing seasons. At the end of the experiment, the total biomass of C-stems and C-needles (formed in G2) was measured after drying for 4 days at 60 °C. The biomass of 100 C-needles was measured from leader shoots.

Needles for all physiological measurements were sampled at the same time from the second and third whorls from different sides of the saplings. The C1-needles (formed during G1) were sampled once in G1 and four times both in D and G2. The C-needles were sampled twice during the follow-up season G2. Potential photochemical efficiency of photosystem II (F_v/F_m) was measured for dark-acclimated (20 min) needles with a portable chlorophyll fluorescence meter (MINI-PAM, Heinz Walz Gmbh, Effeltrich, Germany) as described previously (Repo et al. 2005). The water potential of needles was measured with a pressure chamber within 10 min after the sampling (Scholander et al. 1964). Chlorophyll a (*Chl a*) and b (*Chl b*) concentrations were measured from needles frozen in liquid nitrogen and then stored at −80 °C prior to their analyses (Porra et al. 1989). The needles for soluble sugar and starch concentrations were dried at 40 °C, ground to powder and analysed as in Hansen and Moller (1975).

Changes in the electrolyte balance in needles were measured by electrical impedance spectroscopy (EIS). The impedance spectra (real and imaginary part) were measured at 46 frequencies between 20 Hz and 1 MHz for six C1-needles from each seedling at the same times as other physiological measurements (Repo et al. 1994). A 15-mm-long sample was cut out in the middle of the needle and set between the electrode pastes (Signagel, Parker Laboratories, Fairfield, NJ, USA) connected by Ag/AgCl electrodes (RC1, WPI, Ltd, Sarasota, FL, USA) to the circuit analyser (HP 4284 A, Agilent, Palo Alto, CA, USA). The extracellular resistance (R_e) was estimated on the basis of the parameters of the distributed circuit model (single-DCE) and normalized by the length and cross-sectional area of the sample to get the specific value (r_e, unit Ωm) (Repo et al. 1994).

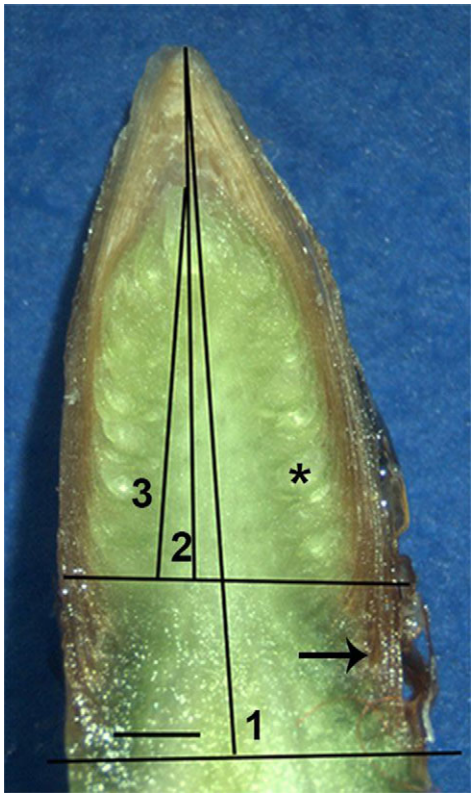

Figure 1. Definition of morphological dimensions of a terminal bud. 1. The height of a bud from the base of empty scales (without dwarf shoots) (indicated with an arrow) up to the top of bud. 2. The height of a bud from the scales with dwarf shoots (asterisk) up to the top of bud. 3. The height of the primordial shoot of the bud from the scales with dwarf shoots up to the top of the apex.

Microscopy of needles, shoots and buds

For anatomical studies, five C-needles were sampled at the end of G2 from the leader shoot and from the middle of the same side shoots as used for physiological measurements. After sampling, the needles were immediately put into the tubes containing the fixative solution (2% glutaraldehyde in cacodylate buffer, 0.05 M pH 7.0). Within 2 days, 0.5- to 1-mm-long cross-sectional samples were cut ~10 mm from the tips of the needles and kept in the fixative for about 22 h. The samples were post-fixed in 1% OsO_4 and further fixed (Kivimäenpää et al. 2001). Cross-sections, about 2 µm thick, were cut on an LKB 2128 Ultratome (Bromma, Sweden) and double-stained, first with 1% toluidine blue and after that with 1% p-phenyldiamine (Sutinen 1987). Each cross-section was photographed with a digital camera (Leica Microsystems CD Camera, Heerbrugg, Switzerland) under a light microscope (Leica Microsystems DM2500, Wetzlar, Germany) with ×10 and ×20 objective magnifications. The length of abaxial and adaxial sides of the needles (together circumference of needle cross section), area of the mesophyll tissue and that of the central cylinder were measured from the photographs. In addition, a number of resin ducts was calculated separately from abaxial and adaxial sides of the needles (cf. Sutinen et al. 2014).

Leader shoots with terminal and whorl buds were sampled for morphological studies at the end of G2. A piece (2.5 cm in length) was cut from the tip of the leader shoot and put into the test tube containing the fixative solutions (see above). Fresh-cut, semi-thin cross-sections were cut with a razor blade from the shoot about 1 cm from the bottom of the terminal bud and photographed with a digital camera at ×6 and ×12 objective magnification under a stereomicroscope. The cross-sectional area of the shoot was measured from the digital images. The number of whorl buds was counted. The terminal bud and the whorl buds of each sample were cut longitudinally into two halves, and the outer and the inner parts of the buds were measured from the photographs taken at ×6 and ×12 objective magnification under a stereomicroscope. The total height of the bud and the height of the primordial shoot were measured, and the number of scales without and with dwarf shoots was calculated (Figure 1).

Statistical analysis

The treatment effects on chlorophyll fluorescence, chlorophyll concentration, soluble sugar and starch concentration, and EIS

parameters were analysed by means of the linear mixed model (procedure MIXED in SPSS Statistics 20.0, IBM Co., New York, NY, USA). The model was $y = \mu + temp + wl + time + temp \times wl + temp \times time + wl \times time + temp \times wl \times time + chamber + \varepsilon$, where temp refers to soil frost treatment, wl to waterlogging treatment, μ is constant and ε is an error term. The treatment and sampling time were regarded as fixed factors, and the chamber was random term. Temp was a fixed main plot treatment (chamber) and wl was a fixed split-plot treatment. The significance of the difference between the treatments at different sampling times was tested by contrasts using Bonferroni corrected significance levels, i.e., the *P*-value of each contrast was multiplied by the number of comparisons at a sampling time. Biomass of the saplings, shoot length and the microscopic measures at the end of G2 was analysed by means of a linear mixed model as described above, except sampling time was excluded. 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 information criteria. Response variables were log-transformed when necessary to fulfil the assumptions of the analyses.

For the root longevity analyses by generalized Kaplan–Meier statistics, the 'interval' package in R (version 3.4.1) was used for short and long roots. The survival probabilities were tested using the 'ictest' function with a Wilcoxon-type test using 999 Monte Carlo replications (weighted exact logrank test) (Fay and Shaw 2010). The appearance and death of individual roots were observed at certain time intervals and the time range to an event (a root death) or to a right-censoring (a root is alive at the last imaging session) was calculated based on those data. Kaplan–Meier curves were plotted using the 'icfit' function of the 'interval' package. The Kaplan–Meier estimator is typically 'undefined' after the last observation if that observation is right-censored (Fay and Shaw 2010), like in our case for all roots that were alive at the end of the experiment. According to Fay and Shaw (2010), this is because the nonparametric maximum likelihood estimation (NPMLE) is not unique in this case, as changes in the survival distribution after that last censored observation do not affect the likelihood of the observed data. The estimator can be undefined also due to non-uniqueness at given intervals. The plotted survival curve is shown as a descending slope over the intervals and as a step function where it is uniquely defined (Fay and Shaw 2010).

Results

Soil and air conditions

Volumetric water content in the mineral soil during dormancy was ~30% without WL, and it increased up to 80% by WL (Figure 2A). By soil freezing, the liquid water content in the mineral soil decreased in Fr treatments. After completion of WL, the soil water content decreased in a few days to 30% in NoFr + WL but that level was reached more slowly in Fr + WL due to the delay of soil thawing. In Fr, soil temperature decreased at the same rate and stayed below 0 °C (minimum −2.5 °C) for about 40 days (Figure 2B). Waterlogged soil thawed and temperature increased at a slower rate in Fr + WL than in Fr + NoWL. The air-saturated soil oxygen content followed changes in volumetric soil water content (Figure 2C). It decreased close to zero by WL if the soil was not frozen (NoFr + WL) but stayed at 25% by soil freezing (Fr + WL). By drainage and soil thawing, oxygen content recovered to 100%. Before the start of the follow-up growing season (Day 176), soil and air temperature was approximately the same in all treatments.

Roots

Short and long root elongation (data not shown) and new root tip formation (Figure 3A) was similar in all treatments during G1. After the treatments in the beginning of the follow-up growing season G2 (start at Day 175), the formation of new root tips (Figure 3A) and production of short roots (Figure 3B) were delayed for about 20 days in Fr + WL. There were two production peaks in short roots in other treatments except Fr + WL but one production peak in long roots only (data not shown). After the short root growth in Fr + WL had started in the latter half of the growing season, the production was higher in Fr + WL than in the other treatments.

At the end of dormancy, the proportion of dead roots ranged from 2% to 10% in short roots and from 0% to 3% in long roots, but there were no significant differences between the treatments (Figure 4). In long roots, the proportion of dead roots increased in Fr + NoWL during the follow-up growing season G2, being higher than in both WL treatments at the end of G2 (Figure 4B). The lowest proportion of dead long roots was found in NoFr + WL. Fine root biomass did not differ between the treatments at the end of experiment (data not shown).

The treatment significantly affected the survival probability of short roots ($P = 0.006$, 99% confidence interval 0.001–0.014) (Figure 5A). Even though it was not possible to assess median root longevity for each treatment, it seemed to be lowest in Fr + NoWL. In short roots, it was lower than expected in Fr + NoWL (test score 18.85) but higher than expected in Fr + WL (test score −23.06). In long roots, the survival probability was lower than expected in Fr + NoWL (test score 18.43).

Shoot growth

Elongation of the leader shoot started and ceased about 7 days later in NoFr than Fr treatments independent of WL treatment (Figure 6A). The final length of the leader C-shoot was not significantly affected by the treatments, and it was 9.4 ± 2.1 cm (mean ± SE) in Fr + WL, 14.4 ± 1.6 cm in Fr+NoWL, 15.7 ± 3.0 cm in NoFr+WL and 16.9 ± 3.5 cm in NoFr+NoWL. No difference was found between the treatments in the initiation and cessation of trunk diameter growth (Figure 6B). Initiation (Day

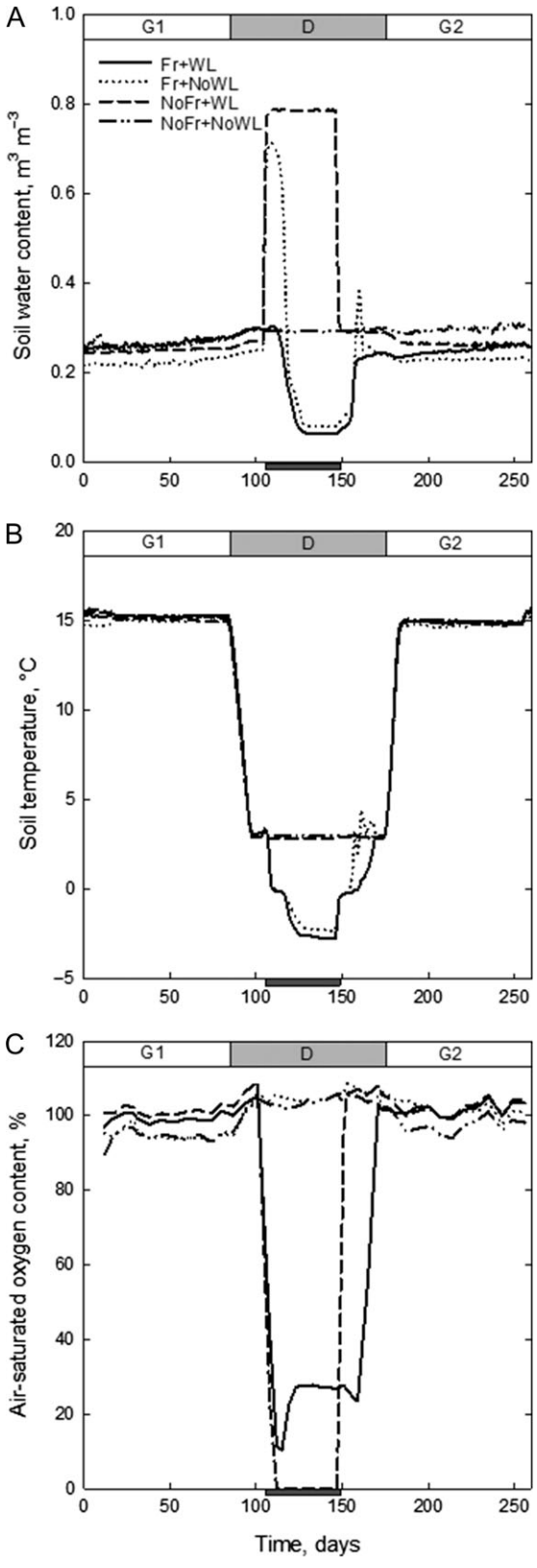

Figure 2. Volumetric water content (A), temperature (B) and air-saturated oxygen content (C) in mineral soil layer before, during and after waterlogging (WL) and soil frost (Fr) treatments (horizontal bar on *x*-axis). NoFr and NoWL refer to no soil frost and no waterlogging, respectively. G1 and G2 refer to growing seasons and D to dormancy. Time indicates days from the beginning of the experiment.

205) and cessation (Day 238) of growth of the needles in the leader shoot was not affected by treatments (data not shown). In the final harvest, the cross-sectional area of the current leader shoot did not differ between the treatments either, being 12.0 ± 2.5 mm^2 (mean ± SE) in Fr + WL, 18.2 ± 5.2 mm^2 in Fr + NoWL, 19.7 ± 5.3 mm^2 in NoFr + WL and 18.5.7 ± 2.3 mm^2 in NoFr + NoWL.

At the end of G2, the biomass of C-needles and C-stems was higher in NoFr than Fr (Figure 7A). The biomass of 100 C-needles of the leader shoot was less in Fr + WL compared with the other treatments (Figure 7B).

Microscopy

Exposure to Fr + WL reduced the circumference and cross-sectional area, as well as the number of stomata rows and that of resin ducts in C-needles of leader shoots (Table 2). The structure of the needles was intact in all treatments. The C-needles in the whorl shoots did not differ between the treatments (data not shown).

Formation of new buds was negatively affected by WL, especially with Fr (Table 3). The outer (P_{WL} = 0.08) and inner (P_{WL} = 0.09) height of leader buds, as well the height of primordial shoots (P_{WL} = 0.06) was shorter and the number of bud scales without (P = 0.03) as well as with the dwarf shoots (P_{WL} = 0.05) was less in Fr + WL compared with Fr + NoWL and NoFr + NoWL (Table 3). In the whorl buds, the outer height (P_{WL} = 0.07) and the height of primordial shoots (P_{WL} = 0.03) were shorter and there were fewer bud scales with dwarf shoots (P_{WL} = 0.04) in WL compared with NoWL, the effect being significant between Fr + WL and Fr + NoWL (Table 3). There were fewer whorl buds (P_{WL} = 0.02) in WL compared with NoWL treatments (Table 3).

Physiological measurements

Dark-acclimated chlorophyll fluorescence (F_v/F_m) of C1-needles was approximately the same in all treatments during both growing seasons (Figure 8A). F_v/F_m decreased in all treatments during dormancy but was significantly lower in Fr than NoFr independent of WL treatment. There was no difference between treatments in F_v/F_m of the C-needles at the end of G2 (data not shown).

The total chlorophyll concentration (*Chl a* + *Chl b*) of C1-needles was lowest during dormancy and increased in all treatments during G2 (Figure 8B). In Fr, the chlorophyll concentrations were significantly higher at one sampling time both during dormancy (end of WL and Fr treatments) and G2 (20 days of G2). There was no difference between treatments in chlorophyll concentration in C-needles at the end of G2 (data not shown).

Water potential in C1-needles decreased during dormancy, and there was a significant main effect for Fr treatment (Figure 8C). Water potential was lower in Fr than NoFr independent of WL treatment (Figure 8C). It increased in all treatments during

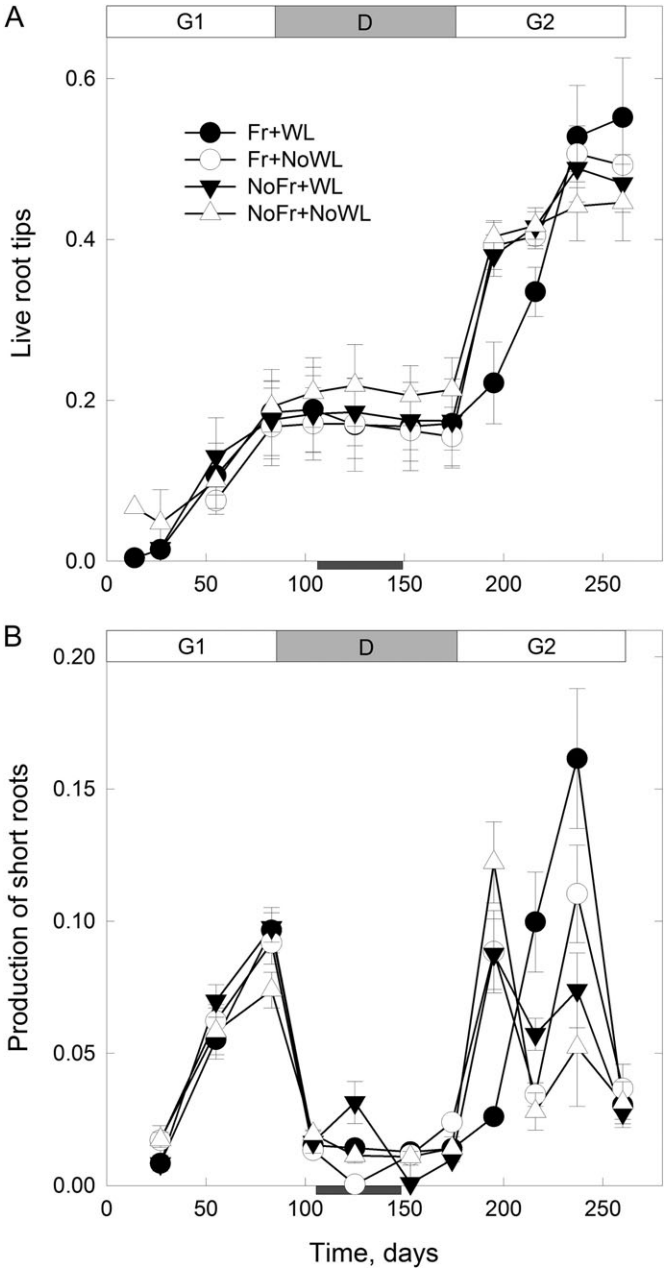

Figure 3. A. Mean number of root tips (A) and production of short roots (B) (both in the Euclidian normalized scale) by minirhizotron imaging in the experiment with waterlogging (WL, df = 3) and soil freezing (Fr, df = 1) treatments (shown by horizontal bar on *x*-axis) of Scots pine saplings during dormancy. The short roots represent the first-order fine roots and the long roots branched, longer fine roots. G1 and G2 refer to the growing seasons and D to dormancy. NoFr and NoWL refer to no soil frost and no waterlogging, respectively. Time indicates days from the beginning of the experiment. df = degree of freedom.

soil thawing, but especially in the Fr treatments, being significantly higher in Fr + WL than in other treatments before the start of G2. During G2, the water potential decreased in Fr, especially in Fr + WL, but the differences between the treatments disappeared towards the end of G2. The treatments had no effects on the water potential of C-needles (data not shown).

Extracellular resistance of C1-needles increased significantly in Fr as compared with NoFr during the treatment period in D and during G2 (Figure 8D). The difference disappeared when the Fr and WL treatments were completed as well as at the end of G2.

The soluble sugar concentration of C1-needles increased during dormancy and decreased during the follow-up growing season, but there were no differences between the treatments (Figure 8E). The starch content of C1-needles decreased towards the end of dormancy without any differences between the treatments (Figure 8F). There was a slight increase of starch content in the beginning of the follow-up growing season G2, especially in Fr treatments. The treatments had no effects on the sugar or starch concentrations of C needles (data not shown).

Discussion

This study shows that soil temperature during dormancy affects the physiology of needles, above- and belowground phenology and growth during the follow-up growing season in the Scots pine. Our hypothesis on the harmful effects of freezing of waterlogged soil was supported by the late initiation of root growth and reduced needle growth as compared with the case with the unfrozen soil. In addition, waterlogging, especially in frozen soil, affected new bud formation in a way that may expose the buds to frost injury during the next winter and thus reduce growth in the next growing season.

Needle physiology followed the annual rhythm as found in the field (Sutinen et al. 2000, Repo et al. 2011). During dormancy, needles showed a shift towards a deeper winter physiology in frozen than unfrozen soil in chlorophyll fluorescence (F_v/F_m), water potential and extracellular resistance. Foliar sugar content, which is also important for frost hardiness (Sutinen et al. 1996, Ögren et al. 1997), did not differ between the treatments here. Reversible reduction in F_v/F_m as found here indicates that thylakoid membranes might be better protected against high photon flux density in frozen, rather than unfrozen, soil in winter (Ottander and Öquist 1991, Sutinen et al. 2000). Furthermore, the water potential was lower and extracellular resistance higher (i.e., less apoplastic water) in Fr than in NoFr, indicating that the Fr-needles are better protected against winter frost compared with NoFr-needles (Repo et al. 2000, Zhang et al. 2002). Our results in experimental conditions suggest that frozen soil during dormancy may increase the ability of needles to stand winter conditions in Scots pine saplings independently of air temperature.

Frost desiccation of needles may injure or even kill 2-year-old seedlings exposed to waterlogging and soil freezing without snow cover in winter (Domisch et al. 2017). Frozen soil with wind and high light irradiance may damage the needles of even mature trees at high elevations (Tranquillini 1982) and of tree seedlings in the nurseries in spring (Heiskanen et al. 2015). In our study, no symptoms of the desiccation injury were observed in the needle anatomy, which may be explained by above 0 °C

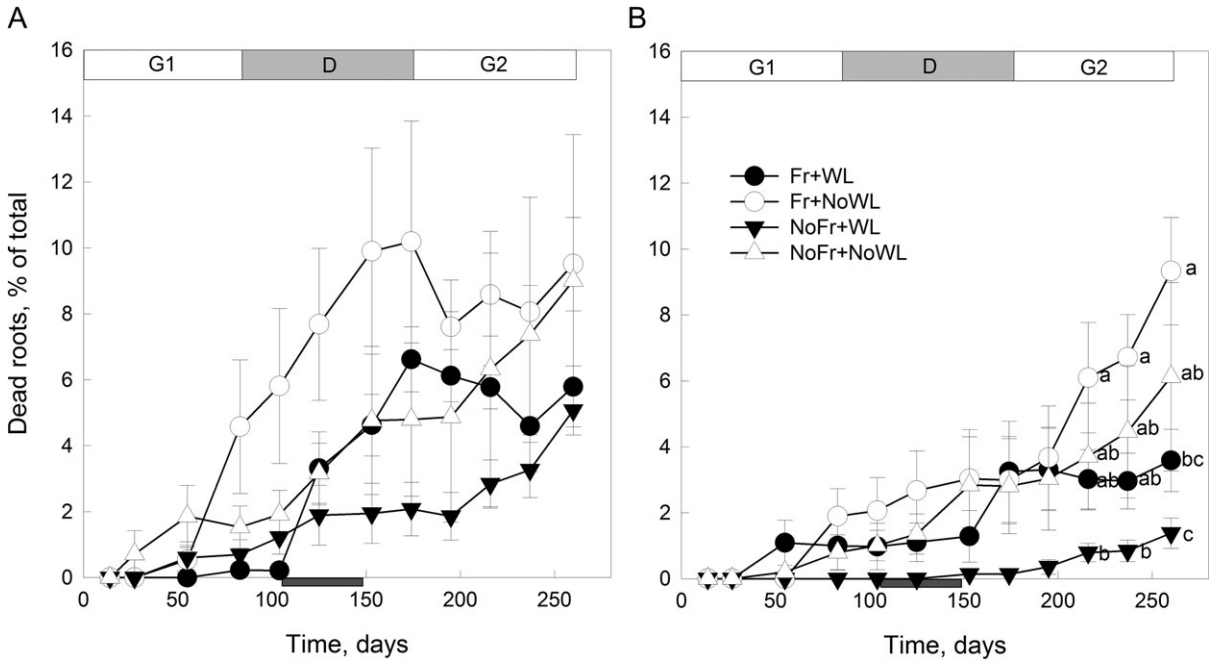

Figure 4. Mean proportion of dead short (A) and long (B) roots out of total by minirhizotron imaging in the experiment with waterlogging (WL, df = 3) and soil frost (Fr, df = 1) treatments (shown by the horizontal bar on x-axis) of Scots pine saplings during dormancy. The short roots represent the first order and the long roots branched, higher than first order fine roots. NoFr and NoWL refer to no soil frost and no waterlogging, respectively, and G1 and G2 to the growing seasons. Time indicates days from the beginning of the experiment. Different letters by sampling times indicate significant differences between treatments ($P < 0.05$). Bars indicate standard errors. df = degree of freedom.

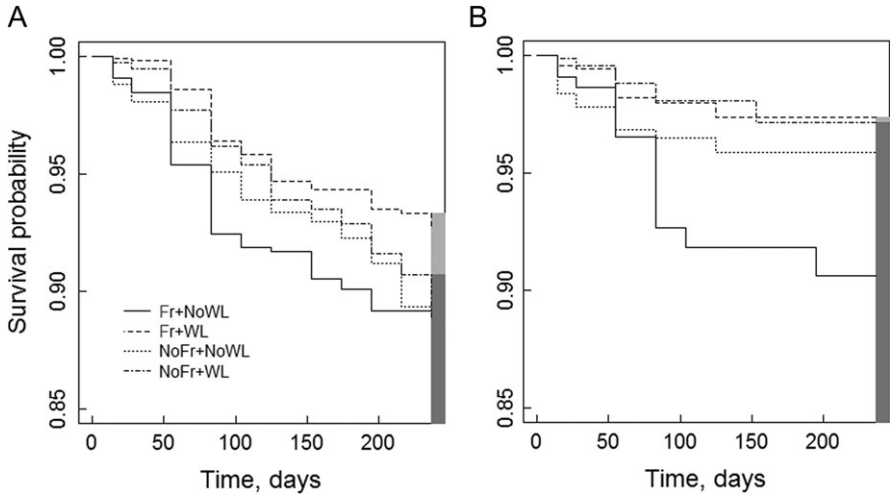

Figure 5. Survival probability of short (A) and long (B) fine roots of Scots pine saplings by Kaplan–Meier statistics in the experiment with waterlogging (WL) and soil frost (Fr) treatments during dormancy. The short roots represent the first order and the long roots branched, higher than first order fine roots. NoFr and NoWL refer to no soil frost and no waterlogging, respectively. The shaded bars on the right indicate that the pattern of the curves is undefined beyond the range indicated in the graphs.

air temperature and reasonable high humidity during the dormancy. Furthermore, the physiology of needles during dormancy in Fr resembled that seen previously in northern Finnish conditions, indicating both the deeper state of photo-inhibition and increased frost hardiness (Sutinen et al. 2000), as supposed to be here too.

The growth started with fine root production, which was followed by shoot elongation, slightly later by stem radial increment and finally by elongation of current needles. The root growth slowed down when shoot growth started. This rhythm is inconsistent with the earlier studies of the Scots pine (Lyr and Hoffman 1967, Iivonen et al. 2001, Konôpka et al. 2005, Schiestl-Aalto

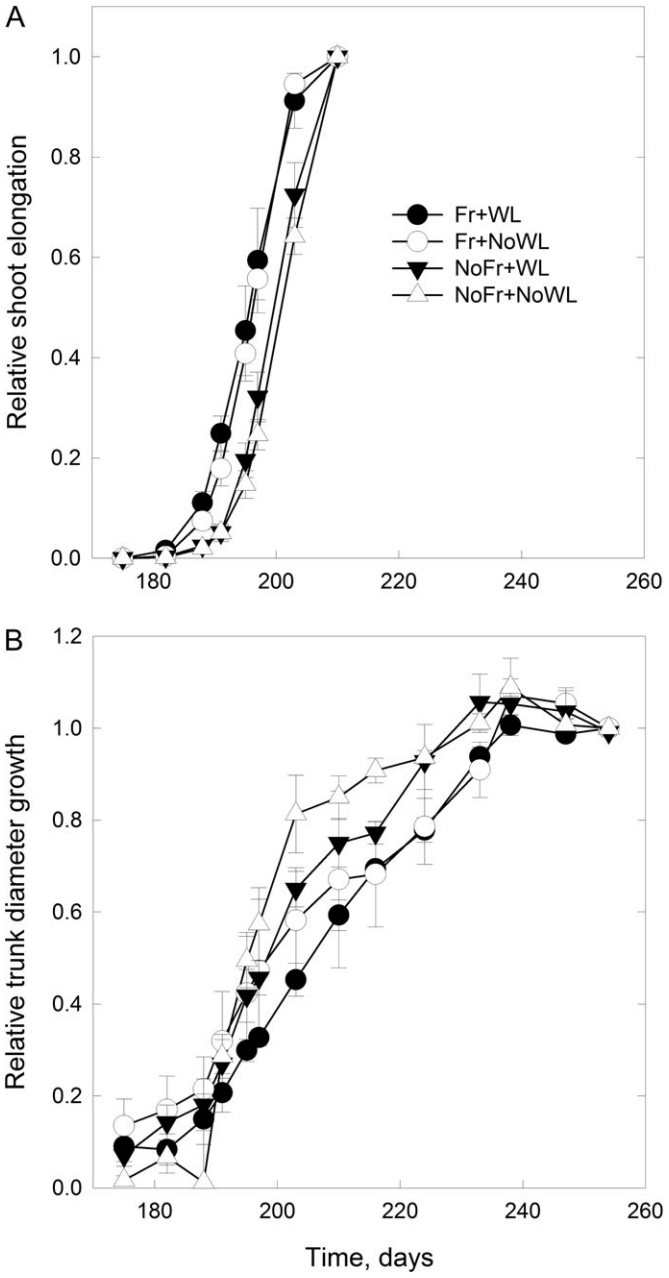

Figure 6. Mean normalized leader shoot elongation (A) and trunk diameter growth 15 cm above the root collar (B) of Scots pine saplings during the follow-up growing season G2 in the experiment with waterlogging (WL, df = 3) and soil frost (Fr, df = 1) treatments during dormancy. NoFr and NoWL refer to no soil frost and no waterlogging, respectively. Time indicates days from the beginning of the experiment. Bars indicate standard errors. df = degree of freedom.

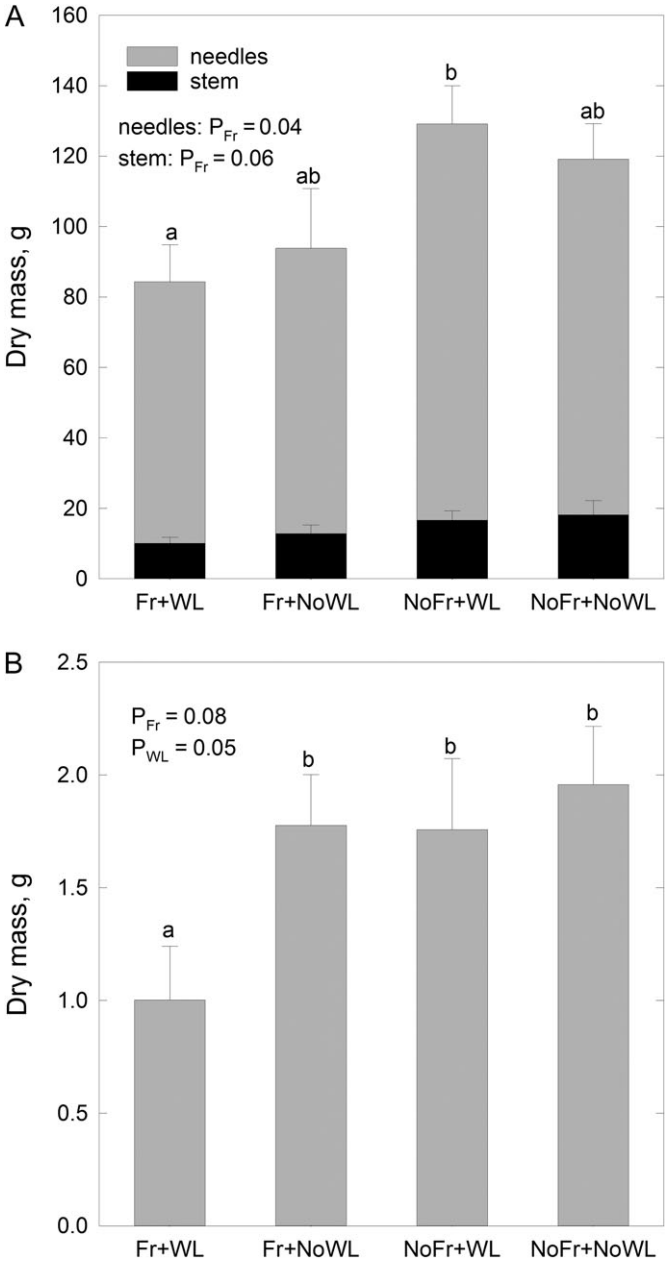

Figure 7. Biomass of current needles and stems (A) and biomass of 100 current needles of the leader shoot (B) of Scots pine saplings at the end of the follow-up growing season (G2) in the experiment with waterlogging (WL, df = 3) and soil frost (Fr, df = 1) treatments during dormancy. NoFr and NoWL refer to no soil frost and no waterlogging, respectively. *P*-values for main effects of Fr and WL treatments are indicated in the figure. Different letters indicate statistically significant differences between the treatments ($P < 0.05$). Bars indicate standard errors. df = degree of freedom.

2017). Short root growth was delayed in Fr + WL in the follow-up growing season, but that condition did not increase the proportion of dead roots, in contrast to Scots pine seedlings exposed to frost heaving (Laiho and Mikola 1964, Sutinen et al. 2014) or to delayed soil frost thawing of Scots pine and Norway spruce up to the growing season (Repo et al. 2005, 2008, 2014). In our case, it is possible that the lower soil temperature up to late dormancy in Fr + WL delayed the initiation of short root growth and root tip formation compared with other treatments, although the soil thawing did not extend until the beginning of the growth season. However, towards the end of the follow-up growing season, short root production recovered and was even higher in Fr + WL than in the other treatments. Similar

Table 2. Morphological characteristics (mean ± SE) of new needles from the leader shoot of Scots pine saplings at the end of the follow-up growing season as affected by soil frost (Fr, df = 1) and waterlogging (WL, df = 3) during dormancy. NoFr and NoWL refer to no soil frost and no waterlogging, respectively. Total circumference is adaxial and abaxial length together. Different letters by rows indicate significantly different treatments ($P < 0.05$), and P_{Fr}, P_{WL} and P_{Fr*WL} significance of the difference between soil freezing and waterlogging treatments and their interaction, respectively. df = degree of freedom.

	Treatment						
Item	Fr + NoWL	Fr + WL	NoFr + NoWL	NoFr + WL	P_{Fr}	P_{WL}	P_{Fr*WL}
Cross-sectional area, mm^2							
whole needle	1.2 ± 0.06	0.82 ± 0.1	1.18 ± 0.08	1.24 ± 0.2			0.09
central cylinder	0.46 ± 0.03a	0.29 ± 0.04b	0.42 ± 0.03b	0.45 ± 0.05b			0.03
Length, µm							
adaxial side	1850 ± 83a	15,015 ± 73b	1877 ± 103a	1979 ± 162a	0.04		0.06
abaxial side	2862 ± 73	2344 ± 128	2810 ± 81	2878 ± 235			0.07
total circumference	4711 ± 155	3849 ± 198	4686 ± 184	4473 ± 393	0.08		0.07
No. of stomata rows							
adaxial side	14.4 ± 1.0a	9.8 ± 0.9b	13.7 ± 0.5a	13.8 ± 1.6a		0.042	0.046
abaxial side	13.6 ± 1.3	10.0 ± 1.1	13.8 ± 0.5	13.8 ± 1.1	0.09		
No. of resin ducts							
adaxial side	2.7 ± 0.4a	1.1 ± 0.1b	2.4 ± 0.4ab	2.2 ± 0.6ab		0.03	
abaxial side	7.8 ± 0.5	5.9 ± 0.2	7.0 ± 0.7	6.8 ± 0.8		0.09	

Table 3. Morphological characteristics (mean ± SE) of leader and whorl buds of Scots pine saplings at the end of the follow-up growing season as affected by soil frost (Fr, df = 1) and waterlogging (WL, df = 3) treatments during dormancy. NoFr and NoWL refer to no soil frost and no waterlogging, respectively. 'out' and 'in' refer to outside and inside dimensions of the buds. *P*-values indicate the significance of the difference between Fr and WL or their interaction. Different letters by rows indicate the significant difference between the treatments ($P < 0.05$). df = degree of freedom.

	Treatment						
Item	Fr + NoWL	Fr + WL	NoFr + NoWL	NoFr + WL	P_{Fr}	P_{WL}	P_{Fr*WL}
Leader buds							
Height, mm							
whole bud (out)	9.8 ± 0.7	6.9 ± 0.7	8.1 ± 0.4	7.8 ± 1.3		0.08	
whole bud (in)	7.4 ± 0.6	5.1 ± 0.6	5.8 ± 0.3	5.8 ± 0.9		0.08	0.09
primord. shoot	5.0 ± 0.5	3.4 ± 0.6	4.0 ± 0.3	3.7 ± 0.6		0.09	
No. of bud scales							
empty scales	7.3 ± 0.8	5.8 ± 0.6	7.0 ± 0.6	5.8 ± 0.5		0.03	
with dwarf shoots	8.5 ± 0.9a	6.0 ± 0.7b	7.8 ± 0.5ab	7.0 ± 0.8ab		0.05	
Whorl buds							
Height, mm							
whole bud (out)	8.0 ± 0.6	5.5 ± 0.9	6.7 ± 0.3	6.3 ± 0.9		0.07	
whole bud (in)	5.8 ± 0.4	3.6 ± 0.7	4.6 ± 0.2	4.5 ± 0.7			
primord. shoot	3.5 ± 0.3a	2.1 ± 0.5b	2.7 ± 0.2ab	2.5 ± 0.5ab		0.03	
No. of bud scales							
empty scales	6.0 ± 0.4	5.7 ± 0.6	6.0 ± 0.3	5.3 ± 0.5			
with dwarf shoots	6.6 ± 0.5a	3.5 ± 0.7b	5.6 ± 0.3ab	5.4 ± 0.8ab		0.04	0.08
No. of whorl buds	5.0 ± 0.5ab	3.5 ± 0.3a	7.8 ± 1.4b	4.5 ± 1.0a	0.1	0.02	

recovery has also been reported in Norway spruce trees (Gaul et al. 2008) and in Scots pine saplings (Sutinen et al. 2014).

In Fr + WL, the elongation of the needles occurred simultaneously with the most intensive period of root growth, resulting in smaller needles and lower foliar biomass as compared with the other treatments. This suggests that, in this case, the roots were stronger sinks for carbon than the needles. The results are indicative that sink–source relation of photosynthesis products between shoots and roots in the growing season is affected by soil conditions during the dormancy (Iivonen et al. 2001, Konôpka et al. 2005, Schiestl-Aalto et al. 2015).

Shoot elongation started earlier in Fr than in NoFr seedlings in both WL treatments. This suggests that more chilling units were accumulated in Fr than NoFr seedlings by frozen soil, but the signal mediation mechanisms from root to shoot remain unknown. According to current opinion, bud burst in spring is mainly regulated by air temperature (Sarvas 1972, 1974, Hänninen 1991, Sutinen et al. 2012), which was the same in all treatments in our

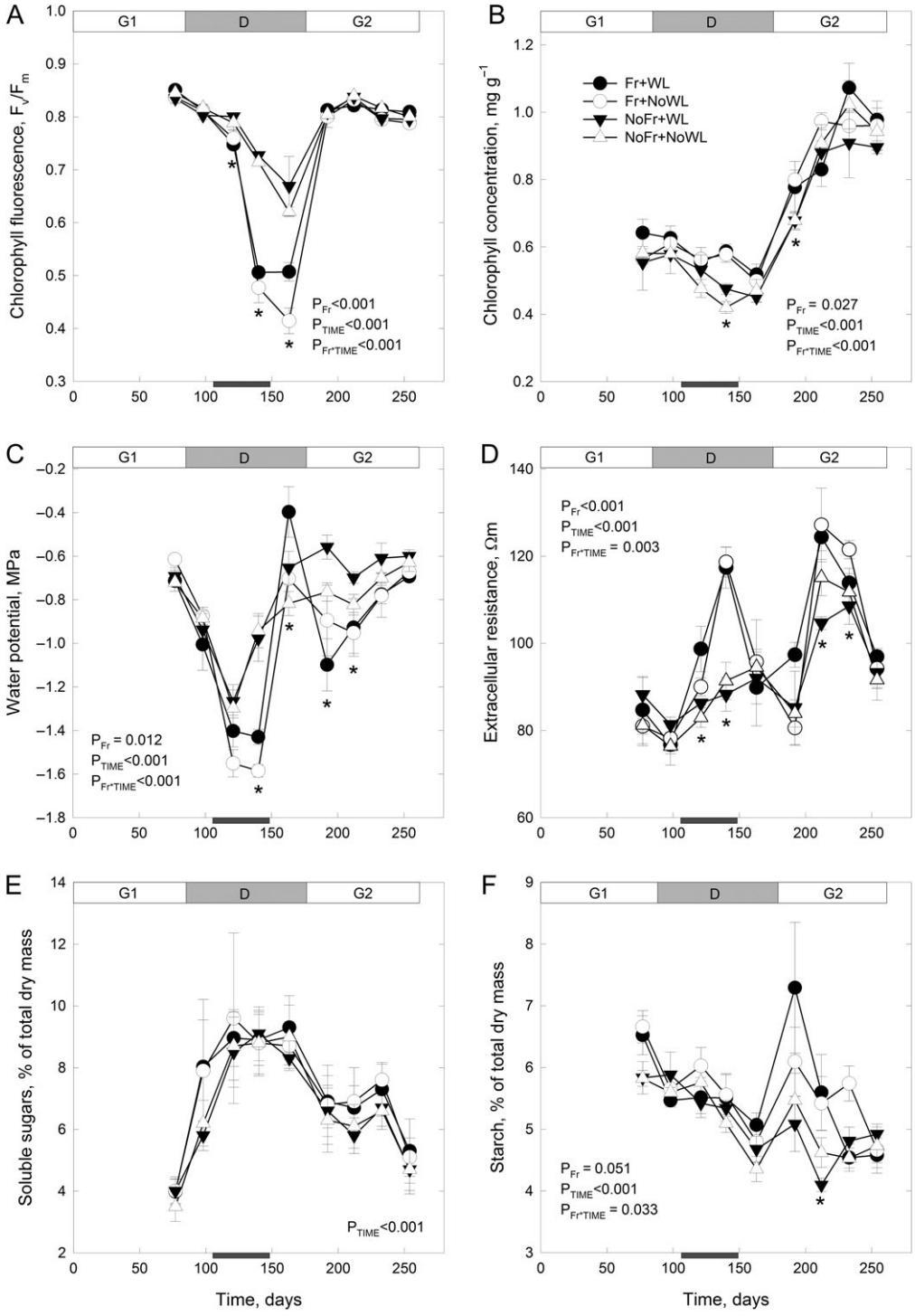

Figure 8. Chlorophyll fluorescence (F_v/F_m) (A), total chlorophyll concentration (B), water potential (C), extracellular resistance (D), and soluble sugar (E) and starch concentration (F) of the C1-needles (formed in G1 season) of Scots pine saplings in the experiment with waterlogging (WL, df = 3) and soil frost (Fr, df = 1) treatments (shown with the bar on *x*-axis) during dormancy, which is the phase between the growing seasons G1 and G2. NoFr and NoWL refer to no soil frost and no waterlogging, respectively. Time indicates days from the beginning of the experiment. *P*-values for the main effects of Fr and time and their interaction are shown in the figure. Asterisks indicate significant differences between Fr and NoFr treatments ($P < 0.05$). Bars are standard errors. df = degree of freedom.

study. As the soil temperature during dormancy seemed to affect the bud phenology in boreal trees, its effect could be worth further study, especially if soil temperature was to remain warm longer in autumn due to climate change. It may be that higher winter soil temperature in the field could increase respiration during dormancy, thus increasing the consumption of stored carbohydrates

in roots also. The lack of carbohydrates may delay the start of shoot elongation accordingly, when several sinks consume photosynthates in spring. Carbohydrate consumption in conifer saplings of boreal forests has been found to increase in mild winters (Ögren et al. 1997).

Biomass of current-year needles was higher in NoFr than Fr treatments. Most of the studies concerning boreal trees have considered air temperature during the current and previous growing seasons as the main driver of growth (Kellomäki et al. 2008, Schiestl-Aalto et al. 2015). Our results showed that wintertime soil conditions should be considered in growth predictions, since there was no difference in air temperature between the treatments but in soil conditions only. The reason for the lower growth due to soil freezing remains obscure but may be connected to a shorter lifetime of fine roots in frozen soil (Laiho and Mikola 1964, Repo et al. 2005, 2014, Sutinen et al. 2014).

Whorl buds of leader shoots were more seriously affected by WL than the leader buds. This is inconsistent with the previous study where Norway spruce trees were exposed to cold soil during early summer (Sutinen et al. 2015). Thus, our results support the idea that the leader buds are the most important and therefore, stronger potential sinks for carbon than whorl buds. However, the reduction of bud scales in both leader and whorl buds, as seen here in Fr + WL, may predispose the buds to winter damage. This occurs because the sink for dehydration of primordial shoot by extraorgan freezing decreases, whereupon its water content remains too high, increasing the risk for intracellular ice nucleation (Ide et al. 1998). The low number of dwarf shoots in Fr + WL will not only decrease the number of future needles but also reduce the elongation of shoots in the following summer. This leads to reduced photosynthetic needle area and a decrease of shoot growth in the following growing season, similar to in Norway spruce exposed to cold soil up to the summer (Sutinen et al. 2015) and in Scots pine where the buds were formed under cold air temperature during previous summer (Schiestl-Aalto et al. 2015).

Conclusions

Soil freezing and waterlogging during dormancy affected below- and aboveground processes of Scots pine saplings. Initiation of fine root growth was delayed by freezing of waterlogged soil. Root growth recovered later in the growing season, but due to the coincidence of the growth of roots and needles, needles remained smaller. Soil freezing without waterlogging decreased the longevity of short and long roots and increased the proportion of dead long roots. Waterlogging, especially together with soil freezing, reduced the number of bud scales predisposing the primordial shoots to frost damage in winter. Shorter primordial shoots and a smaller amount of dwarf shoots and whorl buds would reduce the growth during further growing seasons. We conclude that soil freezing on sites prone to waterlogging should be considered in the management of boreal forests, especially in the face of predicted climate change.

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Conflict of interest

None declared.

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