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An Assessment of Storability of Norway Spruce Container Seedlings in Freezer Storage as Affected by Short-Day Treatment

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Abstract: Determination of safe times at which to transfer seedlings to freezer storage is problematic in forest tree nurseries. The present study aimed to determine the relationship between pre-storage frost hardiness (FH) of different plant parts, dry matter content (DMC), chilling hours (the sum of hours when temperature was between −5 °C and +5 °C), and post-storage vitality, and the impact of short-day (SD) treatment on these relationships. One and a half year old control seedlings and SD-treated seedlings of Norway spruce were transferred to freezer storage (−3 °C) on five occasions during autumn. On each occasion, the FH of buds, needles, stem, and roots, as well as DMC, were determined, and chilling hours were calculated. The vitality of the freezer-stored seedlings was determined through their root growth capacity in the subsequent spring, and through the field performance of the seedlings (shoot growth and seedling damage) at the end of the following two growing seasons. Seedlings were considered to be storable when the FH of the needles was at least −25 °C, and the FH of the roots was about −10 °C in both treatments. Early storage reduced the vitality of the seedlings. SD treatment did not advance the storability of the seedlings, although it alleviated some of the negative effects of early storage by improving the FH of needles and stem, but not that of the roots. The DMC value, indicating storability, was higher for SD-treated seedlings than for control seedlings. When data from five experiments conducted in Suonenjoki were combined, it was found that the relationship between accumulation of chilling hours and needle FH was dependent on nursery treatment and assessment year, which reduces the reliability of using chilling hours in predicting the storability of Norway spruce seedlings. The predicted climate change may complicate the fall acclimation of seedlings. New, user-friendly methods for determining storability of seedlings are urgently needed.

Keywords: chilling unit; dry matter content; field performance; freezer storage; frost hardiness; long-day treatment; *Picea abies*; root growth capacity; storability

1. Introduction

In boreal latitudes, it is common to store conifer seedlings in freezer storage under controlled conditions to avoid the risks present in outdoor storage [1]. These risks include unpredictable and fluctuating weather conditions in winter, and possibly the absence of protective snow cover. The predicted climate change is likely to increase these risk factors in the future [2].

In Scandinavia, the timeframe for transferring seedlings to freezer storage is limited. On one hand, seedlings need to achieve high enough frost hardiness (FH, describes plants ability to tolerate low temperatures) status, which is closely connected with overall stress tolerance, before they are able to tolerate long-term freezer storage in darkness [1]. On the other hand, freezing of soil and seedlings,

as well as accumulation of snow cover, may complicate the handling of seedlings if their transfer to storage is delayed.

Short-day (SD) treatment is a method that is routinely used in nurseries to induce the development of shoot FH in autumn [3]. Although SD treatment improves the FH of shoots, it does not affect the development of FH of roots, which is regulated mainly by temperature: Low soil temperature is required before roots begin to harden [4]. Root growth continues in autumn as long as soil temperature is 2–4 °C [5], and, therefore, the development of FH in roots generally occurs later, and their FH never reaches as low a level as shoot FH does [4,6]. For these reasons, estimating the storability of seedlings based on determining shoot FH alone is risky, and may result in reduced vitality of seedlings in spring caused by root damage [7].

Post-storage vitality and survival of seedlings depend on the pre-storage FH of seedlings, and on the timing and length of storage [8,9]. In Sweden, it is assumed that for one year old Norway spruce (*Picea abies* Karst.) seedlings, shoot FH needs to be about −25 °C to avoid adverse effects of prolonged freezer storage on seedling vitality [10,11], while in British Columbia, a temperature of −18 °C is used to determine the storability of interior spruce (*Picea glauca* (Moench) Voss × *Picea engelmannii* Parry ex Engelm.) seedlings [12,13].

In Scandinavia, freezer storage of Norway spruce seedlings often starts after the middle of October. Due to tight schedules in nurseries, there is pressure to start packing and transferring seedlings to freezer storage earlier than it is traditionally done. Several methods for determining the storability of seedlings in nurseries exist, including measurement of shoot dry matter content (DMC), electrolyte leakage measurements from shoots after freezing exposure [9], a regrowth test [14], and a commercial molecular test [11,15]. DMC increases in autumn due to cell-wall thickening, lignification of the secondary xylem, and accumulation of carbohydrates [16]. Several studies have shown that DMC is a poor indicator of the storability and vitality of conifer seedlings after storage [9,17,18]. According to studies on one year old Norway spruce seedlings, DMC responded rather slowly to changes in growth environment [9], and small changes in DMC resulted in large differences in the storability of seedlings [19]. Despite this, it is common practice in the Nordic countries to base decisions about the timing of seedling storage on determining DMC, because it is a fast method and does not require special equipment or skills. The target value for DMC for storability of one year old Norway spruce seedlings has been determined to be 35–38% [20]. To our knowledge, only a few experiments have been conducted on the applicability of the target value for different seedling batches [21].

In Canada, chilling hours are widely used to determine the storability of conifer seedlings, although the reliability of this method depends on tree species and genotype [8]. An advantage of this method is that it takes into account varying weather conditions in different years. However, the applicability of this method for Norway spruce seedlings is unknown, and more information on the effect of SD treatment on the relationship between FH and chilling hours is needed.

The aim of the present study was to determine the relationship between pre-storage storability indicators and post-storage vitality, and the impact of SD treatment on these relationships. One and a half year old control seedlings and SD-treated container seedlings of Norway spruce were transferred to freezer storage on five occasions during autumn. On each occasion, the FH of buds, needles, stem, and roots, as well as DMC, were determined, and chilling hours were calculated. The post-storage vitality of the freezer-stored seedlings was determined through root growth capacity in the subsequent spring, and through the field performance of the seedlings (damage and shoot growth) at the end of the following two growing seasons. Our hypotheses were as follows: (1) the FH of different plant parts is connected to post-storage growth and survival; (2) reduction of root growth in autumn is connected with FH of roots and storability of seedlings; (3) SD treatment does not affect root FH and, therefore, it does not affect the storability of the seedlings; (4) SD treatment affects the relationship between different storability indicators (FH of different plant parts, DMC, chilling hours).

2. Materials and Methods

2.1. Plant Material

The study was conducted at the Suonenjoki Research Nursery run by the Natural Resources Institute of Finland (62°39′ N, 27°03′ E, altitude 142 m a.s.l.). On 20 June 2011, stand-collected Norway spruce seeds of local origin were sown into hard-walled plastic containers (Plantek PL81F, 81 seedlings per tray, volume: 85 cm³, surface area of a cell: 18.3 cm², growing density: 546 cells m⁻²; BCC, Iso-Vimma, Finland), which were filled with base-fertilized (0.8 kg m⁻³ of 16N:8P:16K soluble fertilizer with micronutrients) and limed (2.0 kg m⁻³) light sphagnum peat (Kekkilä Co., Tuusula, Finland). The seedlings were grown according to Finnish nursery practice for first-year seedlings, using standard fertilization and irrigation procedures [22]. The seedlings were grown in a greenhouse, and in mid-October they were moved to an outdoor growing area, where they overwintered under snow cover and continued to grow the following spring until the SD treatment.

In the second growing season in the nursery (2012), seedlings were irrigated 2–4 times per week and fertilized with 0.1% Forest-Superex solution (22-5-16 for N-P-K + micronutrients; Kekkilä Co., Tuusula, Finland) once per week. Half of the seedlings received SD treatment for three weeks between 16 July and 6 August 2012 (12 h day), which was provided using a blackout curtain. The other half was grown under a natural photoperiod that ranged from 18 h 58 min (16 July) to 17 h 54 min (8 August). Air temperature was measured at the seedling-top level using two Hobo data loggers (type H08-032-08) from 16 July to 29 July 2012. During this period, daily mean temperatures were 15.4 °C for the control seedlings and 16.7 °C for the SD seedlings, and accumulated temperature sums (daily mean temperature >5 °C) were 216 and 234 d.d. (degree days), respectively. After the photoperiod treatments, the seedling trays were randomized into three blocks, with two trays per treatment in each block. Weather conditions for the second-year growing period are shown in Figure 1. Air temperature was collected at a height of 15 cm in the Suonenjoki Research Nursery, starting after the end of the SD treatment. Chilling hours were calculated as the sum of hours when the temperature was between −5 °C and 5 °C.
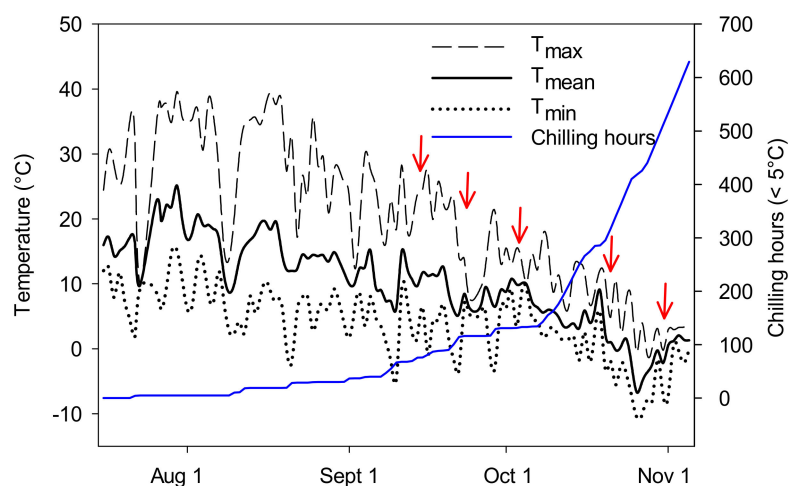

Figure 1. Maximum, minimum, and mean air temperatures and chilling hours (−5 °C to 5 °C) accumulated from the beginning of SD treatment. Temperatures were measured hourly at 15 cm above ground level at the Suonenjoki research nursery, and values of daily averages are shown from the beginning of the SD treatment (16 July 2012) to the last day of freezing exposure (2 October 2012). The dates of the freezing tests are shown by the arrows.

2.2. Determination of Root Growth in Autumn

Root growth was followed by performing a rooting test eight times during the autumn of 2012 (starting dates: 7, 14, and 27 August; 11 and 25 September; 8 and 23 October; and 5 November).

Ten seedlings from both photoperiod treatments (2–3 seedlings from each block and treatment) were used in each test occasion. The seedlings were planted in sand-filled plastic pots (0.75 L) and grown outside under natural weather conditions in a randomized design. The pots were placed next to the seedling trays, described in the previous paragraph, in the nursery. Soil moisture was kept optimal by irrigating the pots with tap water. After a three week growth period, the number and length of roots growing out of the peat plugs were calculated.

2.3. Storing of the Seedlings and Determination of Shoot Dry Matter Content

On five occasions between 12 September and 2 November 2012 (Table 1), seedlings were packed into three cardboard boxes, each containing 63 seedlings per treatment. The boxes were transported to freezer storage (-3 °C). The duration of storage differed between the storage dates (Table 1). Before each freezing test (described below, Table 1), the DMC values of ten randomly selected seedlings per treatment were determined: the upper 2 cm of the shoots was cut and measured for fresh and dry mass (dried at 60 °C for 72 h). The *DMC* was calculated as a percentage:

$$DMC = \frac{Dry\ weight}{Fresh\ weight} \times 100 \tag{1}$$

Table 1. Dates of the freezing tests in autumn 2012, accumulated chilling hours (sum of hours when $-5 \le T \ge 5$ °C) and test temperatures used. Durations of freezer storage for different storage dates are indicated in parentheses.

Date	Chilling Hours	Test Temperatures (°C)	
		Shoots	**Roots**
12 September (245 days)	68	5, −4, −7, −9, −11, −13, −15, −20, and −25	5, −3, −5, −7, and −10
21 September (231 days)	116	5, −5, −10, −15, −18, −21, −24, −30, and −40	5, −4, −6, −8, and −12
5 October (215 days)	144	5, −10, −18, −22, −26, −30, −35, −40, and −55	5, −5, −9, −13, and −17
22 October (198 days)	361	5, −15, −25, −30, −35, −39, −45, −49, and −70	5, −6, −10, −15, and −20
2 November (188 days)	627	5, −20, −25, −30, −40, −45, −50, −60, and −70	5, −8, −13, −17, and −29

2.4. Frost Hardiness of Different Plant Parts

On each of the storing dates (Table 1), the FH of buds, needles, stem, and roots was determined using four air-cooled chambers (WT600/70, Weiss Umwelttechnik GmbH, Reiskirchen-Lidenstruth, Germany). To determine the FH of shoots, 36 seedlings per treatment and block were randomly selected and put into nine trays. One of them served as a control (+5 °C), and the rest of the trays were exposed to eight freezing temperatures on two successive nights, with 12 seedlings per treatment at each temperature (Table 1). The trays were placed in five polystyrene boxes with walls 10 cm thick. To prevent the root plug temperature from dropping below 0 °C at the lowest test temperatures, the peat plugs were heated with a heating cable (Plug'n Heat, 54 W, Ensro, Finland) placed on the bottom of the polystyrene box below the trays. Despite this, the root plugs froze at the lowest test temperatures, and these plugs were thawed at +5 °C before being transferred to a greenhouse.

To determine the FH of the roots, eight seedlings per treatment were exposed to a control treatment (+5 °C) and four freezing temperatures (Table 1). The shoots were covered with a polystyrene box to prevent them from freezing. The target rate of cooling and warming was 5 °C/h, but since the temperatures inside the peat plugs decreased very slowly, the actual cooling rates were slower. Achieving the target minimum temperatures took 13 to 41 h depending on the exposure temperature. The minimum temperature lasted for two to three hours.

After the exposure to freezing, the seedlings were moved to a greenhouse (20 °C or 15 °C, 18 h photoperiod using 400 watt, high-pressure sodium lamps) to study the shoot FH. The seedlings were put into three trays (four seedlings from each test temperature and treatment based on the nursery block randomized within a tray). The seedlings were kept moist by watering them regularly with tap water. After 14 days, frost damage was assessed visually from the stems, needles, and buds. Damage to the needles was scored visually at 10% intervals, and damage to the stems was scored by measuring the length of the damaged part after the stem was dissected. All parts, including buds, were determined to be dead if they had turned brown.

To study the effect of the freezing exposures on roots, a root growth capacity (RGC) test was conducted after each freezing test occasion: Eight seedlings from both photoperiod treatments per freezing test temperature (Table 1) were planted in sand-filled plastic pots (0.75 l), which were moved and randomized to a greenhouse table (20 °C or 15 °C, 18 h photoperiod using 400 watt, high-pressure sodium lamps) and grown there for three weeks. Finally, the number and length of roots grown out of the peat plugs were calculated.

2.5. Vitality of Seedlings after Freezer Storage

On 7 May 2013, the boxes were moved from freezer storage first to +3 °C and then, five days later, to +5 °C. When the boxes were opened, the occurrence of grey mold infections and other seedling damage were evaluated. To investigate the effect of SD and storage date on root and shoot growth capacity after freezer storage, six seedlings per treatment per box (a total of 18 seedlings per treatment) were used to conduct an RGC test (starting dates 20–22 May 2013), using the same method that was used after the freezing tests in autumn.

On 27–28 May 2013, the seedlings were planted in a cultivated experimental field (fine sand with some organic matter). The seedlings were planted in rows, with 0.75 m between seedlings within a row and between the rows. Within each block, there were seedlings from five storage dates and two photoperiod treatments. In each block, five seedlings from each storage box were planted (a total of 15 seedlings per treatment) using a split-plot design (with the storing date as the main plot). The target planting depth was 3 cm (the length of the stem below ground). At planting and at the end of the two subsequent growing seasons (2013 and 2014), height, current-year leader growth (accuracy of 0.5 cm) and stem diameter (accuracy of 0.1 mm) were measured, and the condition of the seedlings was determined: (1) no damage or minor damage, and (2) severely damaged and dead or dying seedlings.

2.6. Relationship between FH of Needles and Accumulation of Chilling Hours—Combined Data from Five Studies

To demonstrate the impact of different nursery treatments and seedling age on the relationship between accumulation of chilling hours and needle FH in Norway spruce seedlings, we combined data from five different studies (including the present experiment). The studies were conducted on one or one and a half year old Norway spruce container seedlings at the Suonenjoki Research Nursery between 2000 and 2017. Needle FH was studied as affected by seedling age and length of photoperiod [23], SD treatment and fall nutrient loading with three different nitrogen levels [24], timing of SD treatment [25], and planting date of SD-treated seedlings [26]. In each of the experiments, air temperature data was measured at a height of 15 cm in the Suonenjoki Research Nursery, and the FH of needles was determined by exposing the seedlings to different freezing temperatures in growth chambers, followed by a growth test and visual analysis of freezing damage in the needles, as described in the above-mentioned publications. The FH estimates and the exposure dates presented in these studies were collected, and the FH was interpolated to each date between exposures. The chilling hours accumulated on the dates when the interpolated FH reached −25 °C in each treatment were used to demonstrate the impacts of nursery treatments and seedling age, as well as the study year.

2.7. Statistical Analysis

All data, except the FH data and the proportion of damaged seedlings and seedlings with grey mold, were tested using SPSS 25 for Windows (SPSS, Chicago, IL, USA) by applying a linear mixed model (MIXED) procedure. *p*-values ≤ 0.5 are reported as "significant", and the list of *p*-values for the main effects of date of storage and photoperiod treatment (and for date of storage × treatment, when available) are shown in Supplementary Table S1. Significant interactions were studied further by conducting a simple main effects test (SME, i.e., post hoc test for interactions) with Bonferroni corrections (*p* ≤ 0.05). The normality of the data and homogeneity of variances were checked from residual plots. Data were ln-transformed to meet ANOVA requirements if necessary. The photoperiod treatment was used as fixed factor for all variables, and, in addition, the storage date was used as a fixed factor for analysis of DMC, root growth data measured after storage, and height and stem diameter growth after the first (2013) and second (2014) growing seasons in the field. Block was used as a random factor for all these analyses. For analyses of field growth data, the storage date within the block and the storage date within the storage date within the block were used as random factors, and the initial height was used as a covariate. The data for the rooting test performed in autumn 2012 and for the RGC data measured in spring 2013 did not pass the tests for normality or homoscedasticity; therefore, the Kruskal–Wallis test, followed by Dunn's post hoc test with Bonferroni correction, was performed.

The effects of the treatments and storage dates on the incidence of grey mold after the freezer storage and the proportion of damaged (including dead) seedlings after the first and second growing season in the field were analyzed using a generalized linear mixed model (GENLINMIXED) in SPSS, using binomial distribution and the logit link function. The fixed and random effects used were the same as used in MIXED models. In the case of the incidence of grey mold, the treatment effect was not significant, and it was excluded from the final model.

The FH values of needles, stems, buds, and roots were analyzed for each treatment and test date using a nonlinear mixed model (NLMIXED) in SAS for Windows 9.4 [27] (Usage Note 56992; SAS Institute Inc., Cary, NC, USA). The proportion of dead buds in each block, and the single observation in each seedling were used in the analysis. The model was

$$
\begin{aligned}
y_i &= \mu + \varepsilon_i \\
&= \left[a + \frac{(d-a)}{1+e^{b(c-x_i)}} \right] + \varepsilon_i
\end{aligned}
\tag{2}
$$

where y_i is the observed value of the i th case of the dependent variable (bud, needle, or stem damage; root number or length), x is the temperature i, parameter d is the upper asymptote (used only for the root number and length; for others it was 1), a is the lower asymptote of the estimated curve, b is the slope, and c is the inflection point of the estimated curve. In the case of roots, the overdispersion parameter γ was added to the model and a generalized Poisson distribution was used (see Usage Note 56549, SAS) [28]. In the other cases, it was assumed that the likelihood is proportional to $\mu^y (1-\mu)^{(1-y)}$ (see Usage Note 56992, SAS) [27]. For a test date, the FH for both treatments was estimated in one analysis. When test dates within a treatment were compared, the FH of all test dates was estimated in one analysis.

We were interested in the temperatures at which the probability of damage was 0.5 (DT_{50}). DT_{50} values were estimated using the equation

$$
DT_{50} = c - \frac{log\left[\frac{(d-0.5)}{(0.5-a)} \right]}{b}
\tag{3}
$$

The statistical significance of the differences between two estimated DT_{50} values was calculated using the delta method and the Wald test statistics described by Lappi and Luoranen (2018) [29].

3. Results

3.1. Effect of SD Treatment on Root Growth of Seedlings in Autumn

Analyses of number and length of new roots grown out of the peat plug gave similar results, and thus, the data are presented only for number of roots. In autumn 2012, the rooting test showed no difference between the treatments in root growth. The sampling date significantly affected root growth, which sharply decreased in August, being close to zero by the end of September (Figure 2).
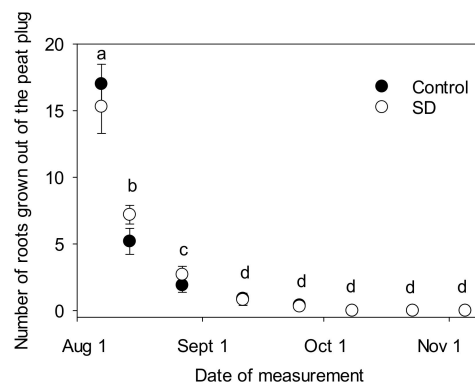

Figure 2. Number of new roots grown out of the peat plug measured in late summer and autumn 2012 from 1.5 year old Norway spruce seedlings that received a short-day (SD) treatment or that were grown under a natural photoperiod (control) (mean of ten seedlings/treatment/date ± standard deviation). Data were subjected to the Kruskal–Wallis test, followed by Dunn's post hoc test with Bonferroni correction. Letters above the symbols denote differences between the dates ($p < 0.05$).

3.2. Dry Matter Content of Shoots and Frost Hardiness of Different Plant Parts

DMC was lower in the control seedlings compared to the SD seedlings, and it increased in both treatments until the sampling performed on 22 October. (Figure 3).
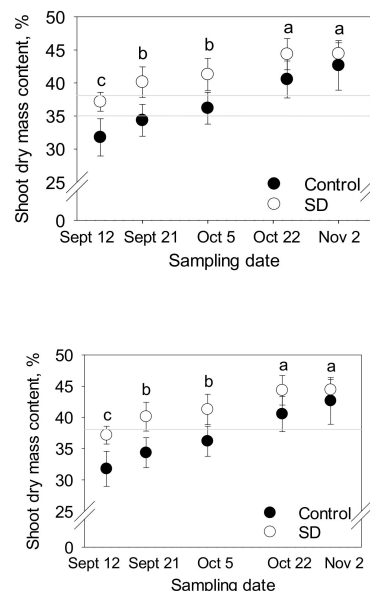

Figure 3. Development of shoot dry matter content (DMC) in 1.5 year old Norway spruce seedlings that received a short-day (SD) treatment or that were grown under a natural photoperiod (control). Values are means of ten seedlings per treatment ± standard deviation. The data were subjected to a linear mixed model (MIXED) procedure. Letters indicate differences between the sampling dates ($p < 0.05$). Gray lines indicate the target values for DMC indicating storability of one year old Norway spruce seedlings (35–38%) according to Rosvall-Åhnebrink (1985) [20].

The FH of the needles, stems, and buds was generally better in the SD seedlings than in the control seedlings, although this difference was not statistically significant for every exposure date (Table 2). SD treatment improved the FH of the roots on only one occasion (22 October). The FH of the needles and stems improved towards the last exposure date, but the FH of the buds fluctuated over the course of autumn. The FH of the roots was rather stable until 5 October, but increased thereafter (Table 2).

Table 2. Frost hardiness (describes plants' ability to tolerate low temperatures), determined as DT_{50} (the temperature causing damage or growth reductions to 50% of the plant part in question) of different plant parts in 1.5 year old Norway spruce seedlings that were grown either under a natural photoperiod (control) or that received a short-day treatment (SD) (mean ± SE, $n = 4$). The data were subjected to a nonlinear mixed model (NLMIXED). Asterisks indicate differences between the treatments within each exposure date ($p < 0.05$). Different letters after the values denote differences between the exposure dates within the treatments ($p < 0.05$).

| Plant Part | Treatment | Date of Freezing Test | | | | |
		12 September	21 September	5 October	22 October	2 November
Bud	Control	−8 (0.5) a*	−14 (0.5) b*	−15 (1.4) bc	−13 (2.0) b*	−19 (7.3) c
	SD	−14 (0.6) a	−22 (0.8) bc	−14 (1.5) a	−19 (1.6) ac	−21 (5.9) ac
Needle	Control	−11 (0.3) a*	−15 (0.4) b*	−24 (1.3) c*	−37 (2.4) d	−42 (0.8) d*
	SD	−20 (1.0) a	−22 (0.6) a	−28 (1.5) b	−45 (4.1) c	−48 (1.1) c
Stem	Control	−11 (0.9) a*	−20 (0.4) b*	−22 (3.0) b	−36 (1.3) c*	−47 (2.1) c*
	SD	−20 (2.3) a	−26 (0.7) b	−24 (3.5) c	−49 (2.4) d	−59 (3.3) e
Root	Control	−8 (0.6) a	−11 (1.0) a	−11 (0.9) a	−14 (1.1) b	−22 (2.3) c
	SD	−9 (0.8) a	−10 (4.8) a	−10 (5.0) a	−18 (1.5) b*	−22 (2.4) b

3.3. Vitality of the Seedlings after Freezer Storage

After freezer storage in May 2013, grey mold symptoms were found in 15% and 3% of the seedlings stored on 12 September and 21 September, respectively, but no mold was observed in the seedlings stored later. Treatments had no effect on the grey mold infections.

The RGC test performed in the spring revealed that SD treatment significantly improved RGC in the seedlings stored on 12 September in comparison with control treatment, and a similar tendency was found on September 21 ($p = 0.091$). In both treatments, root growth of the seedlings stored on 12 September was lower compared to that of the seedlings stored later, and a similar tendency was found in the seedlings stored on 21 September when compared to the later storing dates ($p = 0.059$) (Figure 4).
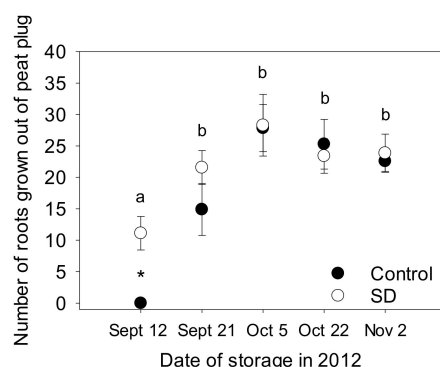

Figure 4. Number of new roots grown out of the peat plug in 1.5 year old Norway spruce seedlings that received a short-day (SD) treatment or that were grown under a natural photoperiod (control). Root growth capacity test was performed in spring 2013 after freezer storage (mean ± SE, $n = 3$). Data were subjected to the Kruskal–Wallis test, followed by Dunn's post hoc test with Bonferroni correction. Letters denote differences between the storage dates ($p < 0.05$). Asterisks denote differences between the treatments within each storing date.

In the first growing season after the planting in 2013, height and stem diameter growth were lower in the seedlings that were stored on 12 September and 21 September, compared to the seedlings that were stored later in autumn. On these storage dates, height and stem diameter growth were higher in the SD seedlings than in the control seedlings (Figure 5A,B). After the second growing season in the field (2014), no clear differences in height growth between the treatments or storage dates were found, but stem diameter growth was still lower in the seedlings stored in September than in the seedlings stored later (Supplementary Figure S1).
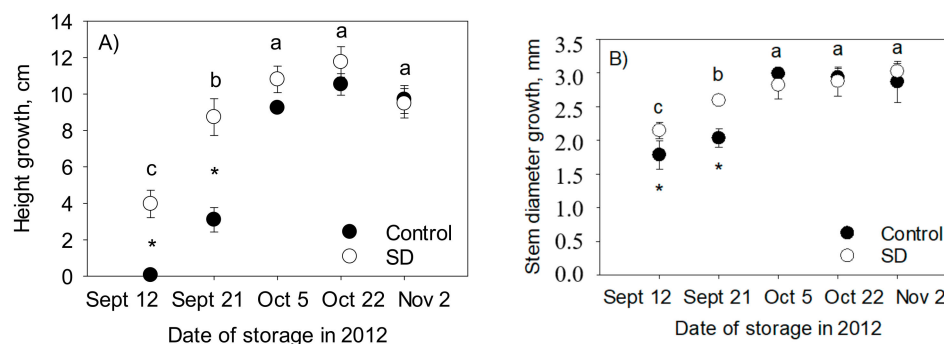

Figure 5. (**A**) Height and (**B**) stem diameter growth of control and short-day (SD)-treated Norway spruce seedlings after the first growing season in the field in 2013 (mean ± SE, $n = 6$ blocks). The data were subjected to a linear mixed model (MIXED) procedure followed by a simple main effects test with Bonferroni corrections. Letters indicate differences between the storing dates, and asterisks indicate differences between the treatments within each storage date ($p < 0.05$).

After the first growing season in the field, 96% and 35% of the control seedlings that were stored on 12 September and 21 September, respectively, were damaged, whereas in the SD treatment, the corresponding values were 20% and 1% (non-significant difference between the sampling dates in SD treatment). On the later storage dates, the proportion of damaged seedlings was rather low in both treatments (Figure 6).
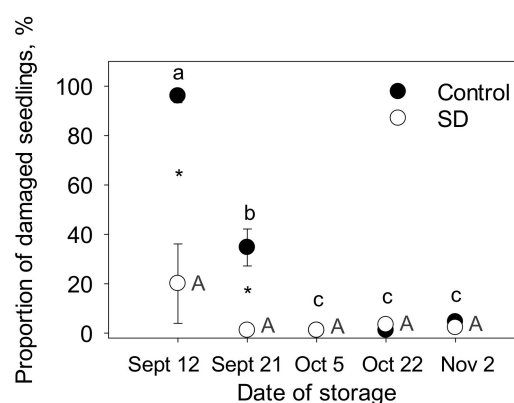

Figure 6. Proportion of damaged Norway spruce seedlings after the first growing season (2013) in the field. 1.5 year old seedlings were grown either under a natural photoperiod (control) or exposed to a short-day (SD) treatment in late summer 2012 (mean ± SE, $n = 6$ blocks). The data were subjected to a generalized linear mixed model. Differences between the storage dates within the control treatment are indicated by small letters, and within the SD treatment by capital letters ($p < 0.05$). Asterisks indicate differences between the treatments within each storage date.

According to the vitality measurements (RGC, and height and stem diameter growth), the first safe storage date for both treatments was 5 October (Figures 4 and 5). There was a strong linear correlation between post-storage RGC and the first-year height growth ($R^2 = 0.932$), and we chose to use the first-year height growth as the measure to describe seedling vitality in further analyses.

3.4. Variables Predicting Seedling Storability

The relationship between the first-year height growth in the field and the variables measured prior to storage was studied to analyze which variables most accurately predicted the storability of the seedlings. In both treatments, seedlings were storable when they had accumulated approximately 130 chilling hours (Figure 7A). At that time, root growth had virtually ceased (Figure 7B) and a rapid increase in root FH was initiated (Figure 8A,B).
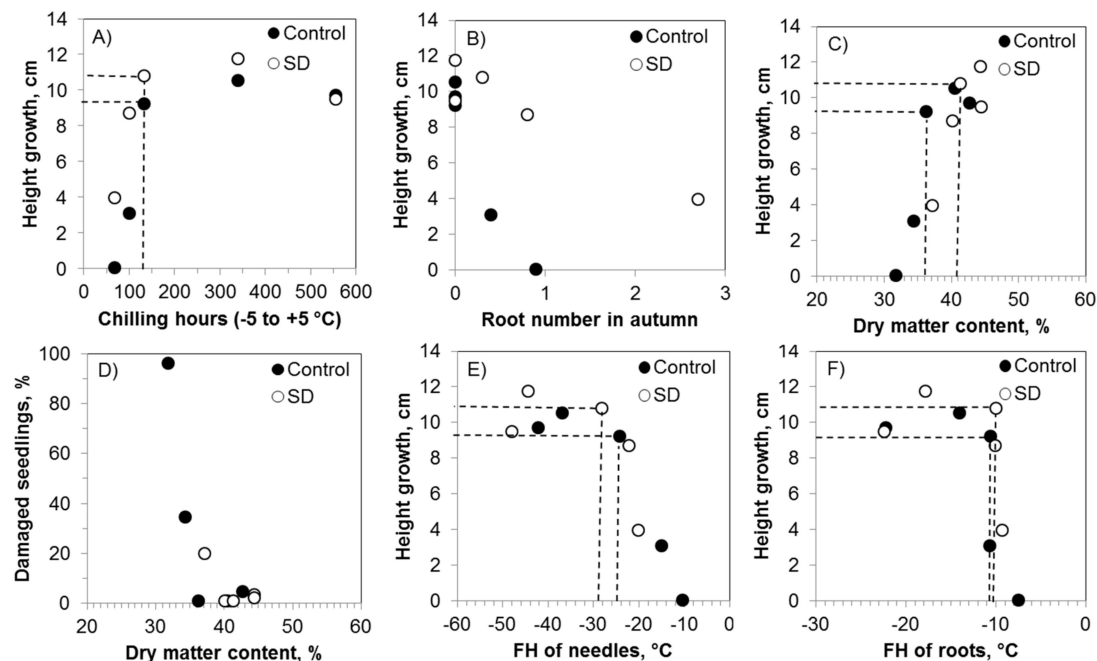

Figure 7. Relationship between height growth at the end of the first growing season in the field (2013) and (**A**) chilling hours, (**B**) root number in autumn, (**C**) dry matter content, (**E**) frost hardiness of needles and (**F**) roots in autumn that were measured on the dates of the freezing tests (Table 1) and used to predict the storability of Norway spruce seedlings that either received short-day (SD) treatment or did not (control). (**D**) The relationship between damaged seedlings at the end of the first growing season in the filed and dry matter content are also presented. FH = frost hardiness (DT_{50}, the temperature at which the degree of damage of needles, stem or buds, or root number was reduced by 50%). Dotted lines indicate the point at which the seedlings reached storability (determined as post-storage root growth potential and first-year height growth; see Figures 4 and 5).

Based on both the proportion of damaged seedlings and height growth after planting, seedlings were storable when DMC values of ~36% were reached in control seedlings, and ~41% in SD seedlings (Figure 7C,D), and when needle FH and root FH was at least −25 °C and lower than −10 °C, respectively, in both treatments (Figure 7E,F). After reaching these FH levels, the hardening of needles and roots sped up (Figure 7E,F). There was a linear increase in needle FH until approximately 130 chilling hours had been accumulated (Figure 8C). The low number of data points made it difficult to estimate how needle and root FH developed between chilling hours 130 and 340 (Figure 8A,C). The FH of the needles increased simultaneously with the DMC (Figure 8D), but the FH of the roots reached a plateau when the DMC was ~37% and 42% in control and SD seedlings, respectively (Figure 8E).
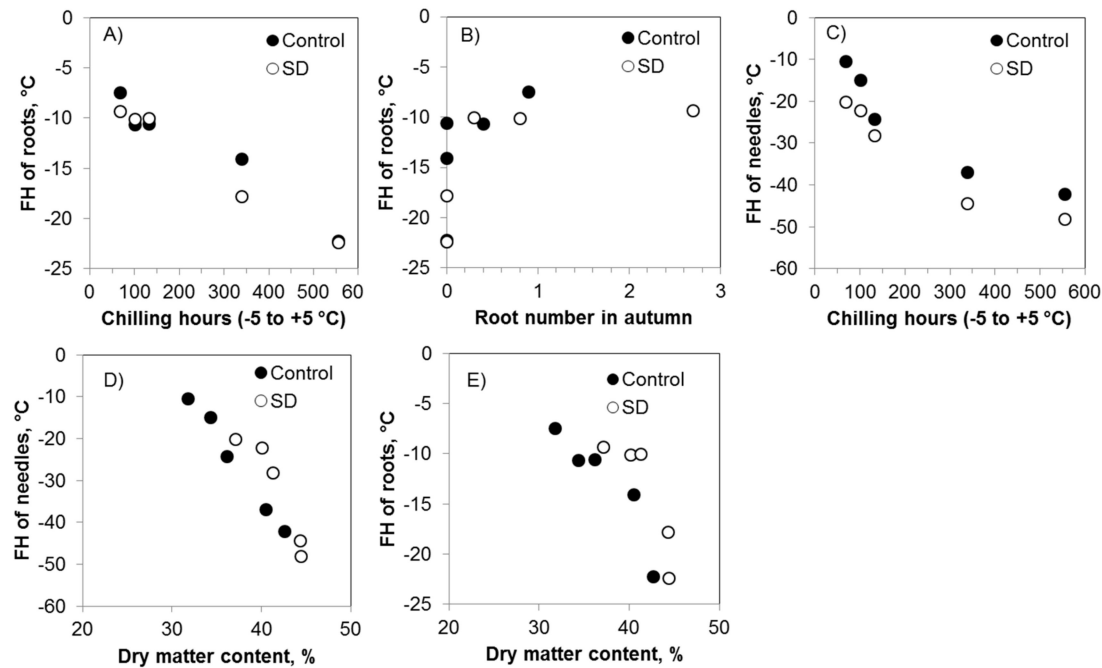
Figure 8. Relationship between (**A**,**B**,**E**) frost hardiness of roots or (**C**,**D**) needles and different variables [(**A**,**C**) chilling hours, (**B**) root number, (**D**,**E**) dry matter content] that were used to predict storability of Norway spruce seedlings that either received short-day (SD) treatment or did not (control). FH: frost hardiness (DT$_{50}$, the temperature at which the degree of damage of needles, stem or buds, or root number was reduced by 50%).

3.5. Relationship between Chilling Hours and FH of Needles

There was heterogeneity in the relationship between accumulation of chilling hours and development of needle FH in one to two year old Norway spruce seedlings when data from five different experiments were combined (Supplementary Figure S2). The chilling hours required to reach the target level of −25 °C for needle FH varied between 45 and 250 chilling hours, depending on the year of the study and nursery cultural practices, such as SD treatment (timing of SD treatment and length of photoperiod) and fall nutrient loading and its interaction with the photoperiod treatment (natural photoperiod or SD) (Figure 9).
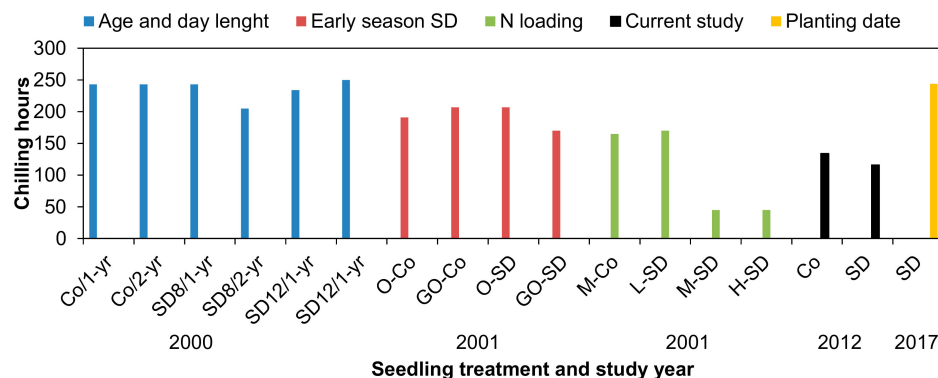

Figure 9. Accumulated chilling hours (sum of hours when temperature was between −5 °C and +5 °C) on the date at which frost hardiness of needles of Norway spruce seedlings reached −25 °C (DT$_{50}$, the temperature causing damage to 50% of the needles). The data from different experiments (indicated by different bar colors) were combined to demonstrate the impacts of various nursery treatments and seedling age on the relationship between accumulation of chilling hours and needle FH. All experiments were conducted in Suonenjoki in different years using the local provenance

and the same growth and FH testing methods. In addition to the present experiment, data were collected from the following studies: Konttinen et al. (2007): one or one and a half year old seedlings were either grown under a natural photoperiod (Co) or had a short-day (SD) treatment (day length either 8 or 12 h, year 2000) [23]; Luoranen et al. (2009): Seedlings grown either outside (O) or in a greenhouse (GO) were exposed to an early-season SD treatment (20 June–11 July, day length 10 hours) or grown under a natural photoperiod (Co) (year 2001) [24]; Luoranen et al. (2008): 1.5 year old control or SD-treated seedlings were nutrient-loaded with low (L), medium (M), or high (H) levels of nitrogen (year 2001) [25]; Luoranen et al. (2019): Needle FH of SD-treated seedlings was assessed in October after the seedlings had been planted in the field in autumn (year 2017) [26].

4. Discussion

4.1. Field Performance of Seedlings

According to the post-storage RGC test and the field performance of the seedlings, it was clear that the seedlings that were stored in September were not ready for freezer storage. The height growth reductions caused by early storage were no longer distinguishable after the second growing season in the field, but reductions in stem diameter growth were still measurable regardless of the treatment. SD treatment did not make seedlings storable at an earlier date compared to untreated control seedlings, although it did alleviate some of the negative effects of the early storage dates on the post-storage vitality of the seedlings. This seemed to be connected with improved pre-storage FH of needles and stem, but not with that of the roots. Improved RGC indicated rapid establishment of the seedlings in the field site [30], which, in our experiment, was seen in higher first-year height and stem diameter growth in the SD seedlings stored in September compared to the control seedlings. Although no severe seedling damage was found in the field in the SD seedlings stored in September, those seedlings suffered from growth reductions, indicating sublethal injury caused by the early storage date. Our results contradicted those of Wallin et al. [21], who found that SD treatment made 1.5 year old Norway spruce seedlings storable at an earlier date compared to untreated seedlings, as indicated by gene expression analysis. The reason for this contradiction may include different temperature conditions during and after SD treatment, which are known to affect development of shoot FH [21,31], or the use of different methods for determining FH and storability. Furthermore, in our experiment, the provenance of the seedlings was more northern than in the study by Wallin et al. [21], and the northern seedlings have been shown to harden earlier [23].

4.2. Frost Hardiness of Seedlings

In our experiment, the FH of the seedlings was assessed by performing a growth test, which is a time-consuming method, but at the same time, it enabled determination of the FH of different plant parts individually. The results indicated that at the time when seedlings were storable, the FH of the needles was at least −25 °C, and the FH of the roots was below −10 °C in both treatments. These values corresponded with values determined for one year old Norway spruce seedlings by using an electrolyte test [10,32]. SD treatment improved needle and stem FH in the course of the autumn, but root FH was only transiently higher in SD seedlings than in the controls (on 22 October).

At the time of root growth cessation in September, root FH was about −10 °C in both treatments, and it rapidly increased afterwards. The needle FH did not reach −25 °C until early October, which may indicate that the reduced vitality of the seedlings stored in September was not caused by low FH of the roots (expressed as DT_{50}, which indicates the temperature at which root growth is reduced by 50%). However, it has been found that even minor reductions (with less than 50% reduction) in root growth may cause declines in the vitality of seedlings, and this was also possible in the case of our experiment. Limited FH of roots has been, in many cases, considered to be the reason for seedling injuries in storage [10,18]. This is based on a generally accepted theory that the FH of roots develops later than that of shoots [4,6], as also shown in our results.

In the root freezing tests, freezing of peat plugs took several hours. The slow cooling rate may have caused root acclimation to lower temperatures than they would have sustained in natural nursery conditions. However, to our knowledge, no information exists on the rate of hardening and dehardening of roots. It is known that buds of Norway spruce can deharden in hours in mid-winter [33].

4.3. Shoot Dry Mass Content as an Indicator of Storability

As expected, the relationship between the DMC and FH of needles and roots was different in the control and SD seedlings: The control seedlings reached higher FH with lower DMC than did the SD seedlings. The seedlings were considered to be storable when the DMC was about 36% and 41% in the control and SD seedlings, respectively. This means that the target values of DMC, suggested by Rosvall-Åhnebrink (1985) [20], could be applied for 1.5 year old seedlings that have been grown under a natural photoperiod, but not for the SD-treated seedlings, which were clearly not storable within this DMC range. The different relationship between DMC and needle and root FH in the control and SD treatments reflects the complex nature of the frost hardening process in conifer seedlings; although the processes of accumulation of DMC and development of FH overlap, their relationship is not consistent, as also suggested by Colombo (1990) [17] and Stattin et al. (2000) [18]. Further, Konttinen et al. (2007) [23] found that this relationship also varies according to the provenance of the seedlings.

4.4. Do Chilling Hours Indicate Storability of Seedlings?

Fall acclimation occurs at lowering temperature after the critical photoperiod has been achieved [34,35]. Therefore, accumulation of chilling hours has been used as a measure to indicate the storability of conifer seedlings, especially in North America [8]. Less information is available on the applicability of the method in predicting the storability of Norway spruce seedlings. According to our results, the seedlings were storable when the chilling hours had reached ~130 in both treatments, with no further increase in post-storage vitality afterwards, whereas needle and root FH continued to increase as the chilling hours accumulated. Because the control and SD seedlings achieved storability simultaneously, and with the same accumulated chilling hours, this method seemed to be a more reliable method for predicting seedling storability than DMC. In principle, this approach of determining seedling exposure to autumnal temperatures could reduce year-to-year variations in seasonal conditions [1]. However, the analysis of the combined data from several studies conducted in different years showed that the chilling hours required to reach the target level of −25 °C for needle FH varied remarkably between 45 and 250 chilling hours, depending on the year of the study and nursery cultural practices, such as SD treatment (timing of SD treatment and length of photoperiod) and fall nutrient loading and its interaction with photoperiod treatment (control or SD) [23–26]. This indicates that storability of Norway spruce seedlings cannot be predicted by any fixed number of chilling hours or by using a certain calendar date, because different seedling batches responded differently to the accumulation of chilling hours in terms of development of FH.

4.5. Storage Duration

In northern countries, it is common to freezer-store Norway spruce seedlings for up to seven months. In our experiment, freezer storage duration varied from six to eight months. The possible impact of this difference on the post-storage vitality of the seedlings cannot be excluded. Long-term freezer storage may cause drought stress and adverse effects on seedling physiology, e.g., through consumption of carbohydrate reserves for maintenance respiration [36,37]. However, in our earlier study on one and a half year old Norway spruce seedlings, extending the freezer storage period from 6 to 8.5 months did not cause alterations in the FH of needles, stem, and buds; concentration of starch and soluble sugars; or incidence of seedling damage. Therefore, the conclusion was made that two year old Norway spruce seedlings can be freezer-stored safely for eight to nine months [38].

5. Conclusions

SD treatment did not make seedlings storable at an earlier date compared to control seedlings, although it alleviated some of the negative effects caused by early storage dates on the post-storage vitality of the seedlings. This was connected with improved FH of needles and stem, but not with that of the roots. Seedlings were storable when needle FH reached −25 °C, and when the FH of roots was above −10 °C. Our results indicated that the target levels for DMC and chilling hours were different for different seedling batches, and therefore, application of these methods for predicting the storability of Norway spruce seedlings may be risky. There is still a need for new, reliable, rapid, and user-friendly methods for determining storability of Norway spruce seedlings in the changing climate.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/6/692/s1, Table S1: Statistical significances of the effects of treatment and date of storage on seedling attributes measured in Norway spruce seedlings that either received short-day treatment or did not. Figure S1: Height and stem diameter growth of control and short-day (SD)-treated Norway spruce seedlings after the second growing season in the field in 2014. Figure S2: Relationship between frost hardiness of needles and accumulation of chilling hours and day of year in Norway spruce seedlings in the data that were combined from five experiments.

Author Contributions: J.L. planned and organized the experiment; J.R. wrote the original draft; J.L. and J.R. performed data analysis and interpretation, reviewed and edited the manuscript and were responsible for the project administration. All authors have read and agreed to the published version of the manuscript.

References

1. Landis, T.D.; Dumroese, R.K.; Haase, D.L. Seedling processing, storage, and outplanting. The container tree nursery manual. In *Agricultural Handbook 674*; Department of Agricultural Forest Service: Washington, DC, USA, 2010; Volume 7, p. 199.

2. IPCC. *Climate Change 2013: The Physical Science Basis*; Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change; Stocker, T.F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M., Eds.; Cambridge University Press: Cambridge, UK, 2013; p. 1535.

3. Landis, T.D.; Tinus, R.W.; McDonald, S.E.; Barnett, J.P. Atmospheric environment. The container tree nursery manual. In *Agricultural Handbook 674*; Department of Agricultural Forest Service: Washington, DC, USA, 1992; Volume 3, p. 145.

4. Bigras, F.J.; Ryyppö, A.; Lindström, A.; Stattin, E. Cold acclimation and deacclimation of shoots and roots of conifer seedlings. In *Conifer Cold Hardiness*; Bigras, F.J., Colombo, S.J., Eds.; Kluwer Academic Publishers: Amsterdam, The Netherlands, 2001; pp. 57–88.

5. Lyr, H.; Hoffmann, G. Growth rates and growth periodicity of tree roots. *Int. Rev. For. Res.* **1967**, *2*, 181–206.

6. Colombo, S.J.; Zhao, S.; Blumwald, E. Frost hardiness gradients in shoots and roots of *Picea mariana* seedlings. *Scand. J. For. Res.* **1995**, *10*, 32–36. [CrossRef]

7. Lindström, A. Freezing temperatures in the root zone—Effects on growth of containerized Scots pine and Norway spruce seedlings. *Scand. J. For. Res.* **1986**, *1*, 371–377. [CrossRef]

8. Grossnickle, S.C.; South, D.B. Fall Acclimation and the Lift/Store Pathway: Effect on Reforestation. *Open For. Sci. J.* **2014**, *7*, 1–20. [CrossRef]

9. Lindström, A.; Stattin, E.; Gräns, D.; Wallin, E. Storability measures of Norway spruce and Scots pine seedlings and assessment of post-storage vitality by measuring shoot electrolyte leakage. *Scand. J. For. Res.* **2014**, *29*, 717–724. [CrossRef]

10. Lindström, A.; Håkansson, L. *The EC-Method—A Way to Assess Storability of Forest Tree Seedlings*; Research Report 95; Swedish University of Agricultural Sciences, Department of Forest Yield: Garpenberg, Sweden, 1996.

11. Stattin, E.; Verhoef, N.; Balk, P.; van Wordragen, M.; Lindström, A. Development of a molecular test to determine the vitality status of Norway spruce (*Picea abies*) seedlings during frozen storage. *New For.* **2012**, *43*, 665–678. [CrossRef]

12. Simpson, D.G. Frost hardiness, root growth capacity, and field performance relationships in interior spruce, lodgepole pine, Douglas-fir, and western hemlock seedlings. *Can. J. For. Res.* **1990**, *20*, 566–572. [CrossRef]

13. L'Hirondelle, S.J.; Simpson, D.G.; Binder, W.D. Overwinter storability of conifer planting stock: Operational testing of fall frost hardiness. *New For.* **2006**, *32*, 307–321. [CrossRef]

14. Mattsson, A. Seasonal variation in root growth capacity during cultivation of container grown *Pinus sylvestris*. *Scand. J. For. Res.* **1986**, *1*, 473–482. [CrossRef]

15. Balk, P.A.; Brönnum, P.; Perks, M.; Stattin, E.; van der Geest, L.J.M.; van Wordragen, M. Innovative cold tolerance test for conifer seedlings. In Proceedings of the USDA Forest Service Proceedings RMRS-P-50, Fort Collins, CO, USA, 13–15 February 2007; pp. 9–14.

16. Levitt, J. *Responses of Plants to Environmental Stress: Chilling, Freezing, and High Temperature Stress*; Academic Press: New York, NY, USA, 1980; Volume 1, p. 510.

17. Colombo, S.J. Bud dormancy status, frost hardiness, shoot moisture content, and readiness of black spruce container seedlings for frozen storage. *J. Am. Soc. Hortic. Sci.* **1990**, *115*, 302–307. [CrossRef]

18. Stattin, E.; Hellqvist, C.; Lindström, A. 2000. Storability and root freezing tolerance of Norway spruce seedlings. *Can. J. For. Res.* **2000**, *30*, 964–970. [CrossRef]

19. Lindström, A. How can we minimize storage damage. In *Seedling Production and Seedling Damage—Conference Held in Uppsala, Sweden 30 January 1996*; Hannerz, M., Ed.; The Forestry Research Institute of Sweden: Uppsala, Sweden, 1996; pp. 34–44.

20. Rosvall-Åhnebrink, G. Preparing seedlings for autumn planting or winter storage. In *Silviculture in Southern Sweden Jönköping 16–17 January 1985*; Eriksson, A., Zimmerman, J., Eds.; Swedish University of Agricultural Sciences: Uppsala, Sweden, 1985; pp. 33–37.

21. Wallin, E.; Gräns, D.; Jacobs, D.F.; Lindström, A.; Verhoef, N. Short-day photoperiods affect expression of genes related to dormancy and freezing tolerance in Norway spruce seedlings. *Ann. For. Sci.* **2017**, *74*, 1–14. [CrossRef]

22. Juntunen, M.L.; Rikala, R. Fertilization practice in Finnish forest nurseries from the standpoint of environmental impact. *New For.* **2001**, *21*, 141–158. [CrossRef]

23. Konttinen, K.; Luoranen, J.; Rikala, R. Growth and frost hardening of *Picea abies* seedlings after various night length treatments. *Balt. For.* **2007**, *13*, 140–148.

24. Luoranen, J.; Lahti, M.; Rikala, R. Frost hardiness of nutrient-loaded two-year-old *Picea abies* seedlings in autumn and at the end of freezer storage. *New For.* **2008**, *35*, 207–220. [CrossRef]

25. Luoranen, J.; Konttinen, K.; Rikala, R. Frost hardening and risk of a second flush in Norway spruce seedlings after an early-season short-day treatment. *Silva. Fenn.* **2009**, *43*, 209. [CrossRef]

26. Luoranen, J.; Pikkarainen, L.; Poteri, M.; Peltola, H.; Riikonen, J. Duration limits on field storage in closed cardboard boxes before planting of Norway spruce and Scots pine container seedlings in different planting seasons. *Forests* **2019**, *10*, 1126. [CrossRef]

27. SAS. *Usage Note 56992: Modeling Continuous Proportions: Fractional and 4- (or 5-) Parameter Logit Models*; SAS Institute Inc.: Cary, NC, USA, 2015; Available online: http://support.sas.com/kb/56/992.html (accessed on 20 May 2019).

28. SAS. *Usage Note 56549: Poisson Regression Using a Generalized Poisson Distribution for Overdispersed Data*; SAS Institute Inc.: Cary, NC, USA, 2015; Available online: http://support.sas.com/kb/56/549.html (accessed on 28 November 2019).

29. Lappi, J.; Luoranen, J. Testing the differences of LT_{50}, LD_{50}, or ED_{50}. *Can. J. For. Res.* **2018**, *48*, 729–734. [CrossRef]

30. Grossnickle, S.C. *Ecophysiology of Northern Spruce Species: The Performance of Planted Seedlings*; NRC Research Press: Ottawa, ON, Canada, 2000; p. 409.

31. Colombo, S.J.; Glerum, C.; Webb, D.P. Winter hardening in first-year black spruce (*Picea mariana*) seedlings. *Physiol. Plant* **1989**, *76*, 1–9. [CrossRef]

32. Lindström, A.; Stattin, E. Root freezing tolerance and vitality of Norway spruce and Scots pine seedlings; influence of storage duration, storage temperature, and prestorage root freezing. *Can. J. For. Res.* **1994**, *24*, 2477–2484. [CrossRef]

33. Räisänen, M.; Repo, T.; Rikala, R.; Lehto, T. Effect of thawing time, cooling rate and Boron nutrition on freezing point of the primordial shoot in Norway spruce buds. *Ann. Bot.* **2006**, *97*, 593–599. [CrossRef] [PubMed]

34. Dormling, I.; Gustavsson, Å.; von Wettstein, D. The experimental control of the life cycle in *Picea abies* (L.) Karst. 1. Some basic experiments on the vegetative cycle. *Silvae Genet.* **1968**, *17*, 44–64.

35. Welling, A.; Palva, E.T. Involvement of CBF transcription factors in winter hardiness in birch. *Physiol. Plant* **2006**, *127*, 167–181. [CrossRef]

36. Ögren, E. Relationship between temperature, respiratory loss of sugar and premature dehardening in dormant Scots pine seedlings. *Tree Phys.* **1997**, *17*, 47–51. [CrossRef] [PubMed]

37. Ritchie, G.A. Carbohydrate reserves and root growth potential in Douglas fir seedlings before and after cold storage. *Can. J. For. Res.* **1982**, *12*, 905–912. [CrossRef]

38. Luoranen, J.; Riikonen, J.; Rikala, R.; Sutinen, S. Frost hardiness, carbohydrates and bud morphology of *Picea abies* seedlings after different lengths of freezer storage. *Scand. J. For. Res.* **2012**, *27*, 414–419. [CrossRef]