From growth cessation to bud burst- conifer seedling development in response to nursery culture and environmental stimuli

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Cover: Norway spruce (*Picea abies* (L.) Karst.) seedlings after SD treatment of different durations.
(Photo: Elisabeth Wallin)

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Abstract

In Sweden, 350-400 million seedlings are produced annually for forest regeneration. About one third of these are overwintered in frozen storage, necessitating accurate methods to assess storability. Young transplants of Norway spruce (*Picea abies* (L.) Karst.) intended for short-term frozen storage were considered storable before reaching target levels for safe storage using shoot dry matter content, freezing tolerance and the molecular test ColdNSure™. Results also indicated that using shoot dry matter content for storability assessment can be misleading, not only for Norway spruce but also for Scots pine (*Pinus sylvestris* (L.)). Post-storage vitality can easily and rapidly be determined by measuring the electrolyte leakage from shoots (SEL) of pine and spruce seedlings. SEL and regrowth tests showed that the vitality of young transplants decreased when the time in storage was prolonged from 3-4 to 5-7 months. Short-day (SD) treatment of seedlings shortens the time for dormancy induction and makes seedlings storable at an earlier date. The activity level of dormancy related genes, and genes associated with freezing tolerance reflects the effect of different treatments e.g., the importance of combining longer periods of SD treatment (21-28 days) with low temperature exposure to rapidly obtain storable seedlings. Gene expression profiles have the potential to be used for assessment of seedling dormancy status, predict the development of freezing tolerance, bud set, the risk for a second bud flush in autumn and the timing of bud burst in spring.

The interest in Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) has increased in Sweden. Frost risks in spring make bud burst timing important when selecting suitable provenances of Douglas-fir for planting. A field trial and a greenhouse study showed the same pattern concerning time of bud burst for a number of Douglas-fir provenances, indicating that greenhouse screening tests can be used for provenance selection. Considering bud burst patterns together with previously reported winter hardening characteristics the interior provenance Three Valley would have a good chance of successful field establishment in southern Sweden.

Keywords: Picea abies, *Pinus sylvestris*, *Pseudotsuga menziesii*, seedling status, gene activity, photoperiod, storability, vitality, transplants, shoot electrolyte leakage

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Från tillväxtavslutning till knoppbrytning – barrplantors reaktioner på några behandlingar i plantskolan och olika temperaturregimer

Sammanfattning

I Sverige produceras årligen 350-400 miljoner skogsplantor och cirka en tredjedel av dessa övervintras i fryslager vilket kräver noggranna metoder för att bedöma lagringsbarheten. Unga omskolningsplantor av gran (*Picea abies* (L.) Karst.) ämnade för kort fryslagring visade sig vara lagringsbara innan de uppnått de gränsvärden för säker långtidslagring som utvecklats för äldre konventionella plantor med avseende på torrsubstanshalt, frystolerans och det molekylära testet ColdNSure™. Resultaten tyder också på att mätningar av torrsubstanshalt kan vara missvisande, inte bara för gran utan även för tall (*Pinus sylvestris* L.). Plantvitaliteten efter lagring kan snabbt fastställas genom att mäta det elektrolytiska läckaget från skotten (SEL) hos tall- och granplantor. Vitaliteten hos omskolningsplantorna sjönk mätt som SEL och vid utvärdering av överlevnad och tillväxt om fryslagringen förlängdes från 3-4 till 5-7 månader. Långnatts(LN)-behandling av granplantor under sensommaren gör plantorna tidigt vilande och lagringsbara på hösten. Effekter av behandlingar kan tydliggöras genom att avläsa aktivitetsnivåer hos några utvalda gener som styr plantornas vila och utveckling av frystolerans. För att uppnå djup vila och tidig lagringsbarhet krävs t.ex. långa LN-behandlingar (21-28 dagar) i kombination med växlande ej för varmt utomhusklimat. Analyser av genaktivitet har potential att användas för att fastställa plantornas vilostatus, förutspå utvecklingen av frystolerans, knoppsättning, risk för en andra skottskjutning på hösten samt förutspå tidpunkten för knoppsprickning nästkommande vår. Intresset för Douglasgran (*Pseudotsuga menziesii* (Mirb.) Franco) har ökat i Sverige. Risken för vårfrostskador gör tidpunkten för knoppbrytning till en viktig parameter vid val av lämpliga provenienser av Douglasgran. Genom att fält- och växthusförsök gav samstämmiga resultat med avseende på knoppbrytning kan växthusförsök vara vägledande och underlätta proveniensval. Mot bakgrund av knoppbrytningsmönster hos testade provenienser samt tidigare studier av invintringsförmåga bedöms inlandsproveniensen Three Valley ha goda etableringsförutsättningar i södra Sverige.

Keywords: *Picea abies*, *Pinus sylvestris*, *Pseudotsuga menziesii*, plantstatus, genaktivitet, fotoperiod, lagringsbarhet, vitalitet, omskolningsplantor, elektrolytiskt läckage

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Dedication

To myself, for finally being ready…

Stirra dig inte blind på en stängd dörr, det kan finnas en öppen dörr bredvid.
Nalle Puh

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Lindström, A., Stattin, E., Gräns, D. & Wallin, E. 2014. Storability measures of Norway spruce and Scots pine seedlings and assessment of post-storage vitality by measuring shoot electrolyte leakage. *Scandinavian Journal of Forest Research* 29 (8): 717-724.

II Wallin, E., Gräns, D., Jacobs, D. F., Lindström, A. & Verhoef, N. 2017. Short-day photoperiods affect expression of genes related to dormancy and freezing tolerance in Norway spruce seedlings. *Annals of Forest Science* 74 (3): art. 59.

III Malmqvist, C., Wallin, E., Säll, H. & Lindström, A. 2017. Differences in bud burst timing and bud freezing tolerance among interior and coastal seed sources of Douglas fir. *Trees* 31(6): 1987-1998.

IV Wallin, E., Gräns, D., Stattin, E., Verhoef, N., Mikusiński, G. & Lindström, A. Evaluating methods for storability assessment and determination of vitality status of Norway spruce transplants after frozen storage. (Manuscript)

The contribution of Elisabeth Wallin to the papers included in this thesis was as follows:

I Anders Lindström and Eva Stattin conceived and designed the experiment and carried out the measurements. Elisabeth Wallin together with Daniel Gräns, Anders Lindström and Eva Stattin analyzed the data and wrote the manuscript.

II Elisabeth Wallin performed all experimental work, analyzed the data and was responsible for writing the manuscript. Co-writers of the manuscript were Daniel Gräns who also contributed in data interpretation together with Anders Lindström who supervised the work. Nathalie Verhoef was responsible for gene activity analyzes. Douglas .F. Jacobs participated in data interpretation and paper writing.

III Elisabeth Wallin together with the first author Cecilia Malmqvist designed and carried out the experiment as well as analyzed the data, conducted the statistical analyses and wrote the paper with support from Anders Lindström and Harald Säll.

IV Elisabeth Wallin conceived and designed the experiment together with Anders Lindström and industrial partners involved in the project. She also performed all experimental work, analyzed the data and was responsible for writing the manuscript. Co-writers of the manuscript were Daniel Gräns, Eva Stattin, Nathalie Verhoef, Grzegorz Mikusiński and Anders Lindström.

Abbreviations

DMC	dry matter content
EC	electric conductivity
EL	electrolyte leakage
FAO	Food and Agriculture Organization of the United Nations
RGC	regrowth test by measuring root growth capacity of seedlings
SD	short-day treatment
SEL	shoot electrolyte leakage
$SEL_{diff-25}$	storability test by measuring the isolated effect of freezing shoots to -25°C

1 Introduction

1.1 Toward better quality control of seedlings

More than two thirds of the Swedish land area is covered by forests according to the Food and Agriculture Organization of the United Nations (FAO) (FAO, 2017), which makes Sweden one of the most forested countries in the world. Commercial forest production is very important, and Sweden has a long industrial history for which the access to forest has played a major role. A good example is the early development of mining and metal processing, which demanded large amounts of wood (Kardell, 2003). Also today, the forest industry is of major importance to the Swedish economy (Statistiska Centralbyrån, 2017).

Already in 1903 the Swedish state governed reforestation by law and the main purpose of the first forestry act was to ensure reforestation of harvested areas (Enander, 2003). Fulfilling the legal requirements for an approved new forest generation could be done either by natural regeneration, sowing or planting (Skogsstyrelsen, 2017a). Today planting dominates, covering about 80% of the annual regeneration area. Approximately 350 – 400 million seedlings of mainly Norway spruce (*Picea abies* (L.) Karst.) (53%) and Scots pine (*Pinus sylvestris* L.) (42%) are planted annually in Sweden, on a total area of about 150 000 - 160 000 hectares (Skogsstyrelsen, 2017b). A standard sized nursey in Sweden produces about 20-25 million seedlings annually. The seedling production is mainly (86%) focused on container 1-1.5-year-old seedlings, but there is also a small amount (14%) of bare root seedlings (Skogsstyrelsen, 2017c). For nurseries, to be able to deliver seedlings of high quality, there is a need for knowledge about how seedlings react to various environmental stresses. Seedling quality was described by Lavender et al. (1980) as “fitness for purpose”. Nurseries aim to produce vital seedlings, and there is a possibility to

predict seedling field performance by assessing certain quality attributes (Mattsson, 1996; Grossnickle and South, 2017). Seedling vitality is a part of the quality concept and possibly its most important component.

A number of negative factors in the nurseries can affect seedling vitality, for example sub-optimal growth conditions, or exposure to extreme thermal or light conditions. Outdoor winter storage of containerized seedlings is generally a risky venture. Fluctuating or very low temperatures in combination with lack of snow cover can be detrimental for seedlings stored outdoors. According to Lindström (1986), root damage is one of the main reasons for deteriorated vitality of outdoor stored seedlings. Seedlings stored indoors in cold or frozen storage have the advantage of being kept in a temperature controlled environment. However, if the seedlings are not prepared for storage, their vitality will be negatively affected and the damages could be lethal. It is well known that in order to be ready for low winter temperatures, seedlings have to go through a number of physiological changes to be able to withstand intercellular freezing (see e.g. Levitt, 1980; Bigras and Colombo, 2001). Therefore, it is important to have access to reliable methods that can predict storability during winter and post-storage vitality so that the nurseries can be sure of delivering high quality seedlings for planting. These are important questions that this thesis deals with for Norway spruce (Study I, II, and IV) and Scots pine (Study I). Besides questions linked to storability and vitality this thesis also deals with variations in the pattern of bud burst in spring between different provenances of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings and a local Norway spruce provenance (Study III). This is an important issue concerning the quality of seedlings as early bud burst increases the risk for frost damages in the field.

Swedish nurseries have recently started to produce minor quantities of late sown containerized transplants and this assortment is expected to increase in the future. These seedlings are normally in a very young stage at the time of storage. A part of this thesis (Study IV) aimed to evaluate whether the common methods used for determining storability and vitality of conventional older seedlings also can be applied to these young transplants (see Study IV).

1.2 Shortened photoperiod promotes growth cessation and bud set

The vegetative phase of conifer seedling growth is influenced by changes in the photo- and thermo-period (for references see Grossnickle, 2000). It is well known that night length is the major environmental factor that promotes growth cessation and induces the development of dormancy (Dormling et al., 1968;

Leikola, 1970; Heide, 1974a; Aronsson, 1975; Ekberg et al., 1979). The night length needed for bud set of conifers is related to both latitude and elevation (Grossnickle, 2000). For example, northern Norway spruce provenances require a shorter night length for initiation of bud set compared to provenances from southern latitudes (Dormling and Lundkvist, 1983). Bud set also occurs at shorter night lengths for seed sources from higher elevations, compared to low-elevation areas (Grossnickle, 2000). Even if dark hours is considered the most important factor for growth cessation, decreasing temperatures also triggers bud set as well as dormancy induction during fall (Dormling et al., 1968; Grossnickle, 2000). At the time when terminal buds have formed on shoots, spruce species are generally considered dormant (Grossnickle, 2000) and seedlings start to develop freezing tolerance (Bigras et al., 2001).

At forest tree nurseries in Scandinavia and across Canada it is common practice to use short-day (SD) treatment by using black curtains to promote early growth cessation and induce bud set (Dormling et al., 1968; Heide, 1974a; Ekberg et al., 1979; Colombo et al., 1989; 2001; Flöistad and Granhus, 2013). Also, this method is known to increase seedling shoot freezing tolerance (Dormling, 1982; Colombo et al., 1989; 2001; Jacobs et al., 2008). The SD treatment can be performed in greenhouses or outdoors in specially equipped areas. The duration of SD treatments varies widely between nurseries and regions, but normally falls within the range of 14-35 days, with a photoperiod of 8-12 h (Rosvall-Åhnebrink, 1982; Konttinen et al., 2003; Jacobs et al., 2008; Luoranen et al., 2009; MacDonald and Owens, 2010; Flöistad and Granhus, 2013). If SD treatment is too extensive, however, photosynthesis can be reduced which may lead to a decline of seedling growth and vigour. To avoid reductions in root mass and shoot diameter of Douglas-fir MacDonald and Owens (2010) recommended SD treatment lasting for 3 weeks instead of 4-6 weeks. However, too short of a SD treatment may not have the desired effect i.e. speeding up growth cessation and development of freezing tolerance. Therefore as described in Study II it is important to identify effective and reliable tools or measures that can indicate when the seedlings have reached a status where further SD treatment is not needed. Flöistad and Granhus (2013) evaluated the effect of different lengths (7, 10, 14 and 17 days) of SD treatment with a photoperiod of 10 h, and the results showed that even the shortest (7 days) SD treatment resulted in height growth cessation of Norway spruce seedlings. This indicates that genes related to dormancy induction could be activated already after a period of only 7 days of SD treatment. Mapping the gene expressions after SD treatment (see Study II), could therefore help to explain the known physiological changes that occur when seedlings are exposed to short days.

As mentioned above, there are several studies showing physiological changes in Norway spruce seedlings as an effect of different SD treatments, but to my knowledge, few previously studies (Stattin et al. 2011) have been presented concerning how the activity of dormancy related genes changes as an effect of different lengths of SD treatment. For Norway spruce, Stattin et al. (2011) identified dormancy-related genes that respond to SD treatment, and we found an opportunity to use these sets of genes as a tool to measure dormancy development during ongoing SD treatment (see Study II).

1.3 Bud burst in spring

Bud development of conifers starts in summer with bud formation followed by bud set in autumn. Afterwards, the seedlings go through several developmental stages before bud burst appears in spring. The chilling requirements for dormancy release, which is a prerequisite for further growth, differs between species and provenances (Dormling et al., 1968; Grossnickle, 2000). The required cumulative chilling hours (<+5°C) needed to break the dormancy state vary for different spruce species (Ritchie, 1985; Grossnickle, 1989; 2000; Silim and Lavender, 1991; Hannerz et al., 2003).

Temperature (Dormling et al., 1968; Grossnickle, 2000) and photoperiod during growth cessation and bud set (Dormling et al., 1968; Heide, 1974a; Sögaard et al., 2007; 2009; Flöistad and Granhus, 2010) are important factors affecting the timing of bud burst in spring. However, it is well known that the temperature accumulation (sensu Hannerz, 1994b; Sutinen et al., 2012) during spring is the major factor affecting the time of bud burst. To describe the impact of temperature during spring the temperature sum is normally used, which is the accumulated daily mean average temperature above +5°C. When certain threshold values are reached the buds start to swell and the bud burst process starts (Sarvas, 1972; Hannerz, 1994a; 1994b; 1999). For Norway spruce the timing of bud burst differs between provenances with an earlier bud burst for northern, compared to southern provenances (Heide, 1974b; Hannerz, 1994b; Morén and Perttu, 1994; Hannerz, 1999). To meet the anticipated warmer climate in Sweden, the interest in Douglas-fir to be used for forest regeneration has increased (Malmqvist, 2017). Douglas-fir seedlings have been planted in Sweden since the early 1900s (Martinsson and Winsa, 1986), but to a very small extent, and only in the very southern parts. The reason for the limited use of Douglas-fir seedlings in Sweden is mainly the lack of suitable provenances that can withstand spring frost and early autumn frost occasions (for references see Malmqvist, 2017). One way of determining provenances of Douglas-fir suitable

for planting in Sweden could, as a complement to field trials, be to perform experiments in greenhouse. In this controlled environment timing of bud burst of different provenances can be studied and compared with a local provenance of Norway spruce as a reference (see Study III). A possible way to ensure safe storage of Douglas-fir seedlings could be to use SD treatment, but this may result in an earlier bud burst during spring as shown for Norway spruce by Konttinen et al. (2003). Mapping the expressions for dormancy related genes could help to forecast the timing of bud burst for conifer seedlings (see Study II).

1.4 Freezing tolerance, storability and post-storage vitality

For safe long term cold or frozen storage, seedlings need to be classified as storable according to tests available for forest nursery practice. Measurement of dry matter content (DMC) from shoots (Rosvall- Åhnebrink, 1985) is commonly used in nurseries for assessment of seedling storability. However, there are studies indicating that the DMC-method in some cases can be misleading (Colombo 1990; Lindström 1996). A more accurate method is to assess seedling storability by measurements of shoot freezing tolerance and good correlations were shown between tolerance to low temperature exposure (e.g. -25° C) and seedling ability to withstand long term frozen storage (5-7 months) (Lindström and Håkansson, 1996; Colombo, 1997; Brönnum, 2005; L'Hirondelle et al., 2006). Freezing tolerance is usually measured by determining the electrolyte leakage from shoots after freeze exposure (Aronsson and Eliasson, 1970; L'Hirondelle et al., 2006).

During the last 10 years the molecular test ColdNSure™ (Joosen et al., 2006; Balk et al., 2007; Balk et al., 2008; Stattin et al., 2012) has been commercially used by Swedish nurseries for assessment of seedling storability. Joosen et al. (2006) and Balk et al. (2007) found that gene expression from freezing tolerance related genes was as good as low temperature (-25°C) induced electrolyte leakage in predicting storability. The ColdNSure™ test is more rapid and easy to perform compared to freezing tests (Stattin et al., 2012).

The accuracy of DMC as a tool for determining storability of different spruce species has previously been reported by Colombo (1990) and Lindström (1996), but no studies have so far been done on Scots pine in this respect (see Study I). Also, there is a need to further investigate the ability of different storability measurements to describe short term changes in storability due to e.g. warm temperatures in late autumn (see Study I). As the methods for determining seedling storability (the DMC, freezing tolerance and ColdNSure™ methods)

are all based on studies performed on conventional 1-1.5-year-old seedlings, there is need to investigate if these methods can be applied to late sown, young, containerized transplants (see Study IV).

Conventional seedlings can successfully be frozen stored for a period of 5-7 months if they have reached the status of being storable. Even though seedlings are ready for storage by developing e.g. tolerance to low temperature exposure, they can still become severely injured by e.g. imperfect storage environments and packaging. Access to easy and reliable methods for determination of seedling vitality status after frozen storage is important for forest tree nurseries. To assess vitality of containerized seedlings the well-known RGC test (Mattsson, 1986; 1991) is still used as a regrowth test to measure root growth capacity and survival by many nurseries. However, a disadvantage is that the method is rather time consuming. Besides regrowth tests, there are several methods that can be used for detecting damaged seedlings (for references see Stattin et al., 2012). Stattin et al. (2012) showed that is it possible to use the expression of selected vitality related genes to mirror the vitality status of seedlings. McKay (1992) presented a method for assessment of post-storage seedling vitality by measuring electrolyte leakage (EL) from roots of bare root seedlings. According to McKay (1992) high values of EL from roots correlated with poor seedling establishment in the field. However, it has been shown that this method cannot be used for containerized seedlings, as electrolytes from roots can diffuse out in the surrounding substrate without being discovered (Repo and Ryyppö, 2008). In shoots, however, severe freezing damages can be detected by high electrolyte leakage levels from injured cells (Aronsson and Eliasson, 1970; L'Hirondelle et al., 2006). This fact together with observations that fungal infections can result from high levels of leakage (Landis 1989; Capieau, 2004) due to stresses that affect cell structure, led to the question as to whether the natural leakage from shoots could be used as a test of seedling vitality during or after storage (see Study I, IV). An advantage to such a test is that it is rapid and easy to perform with rather simple equipment.

1.5 Young transplants

In order to facilitate more efficient use of greenhouses and storage environments, a new type of very small containerized seedlings intended for transplanting has recently been introduced (Mattsson, 2016), and in 2017 approximately 42 million young transplants were produced in Swedish nurseries (A. Lindström, personal communication, December 1, 2017). Seeds are sown in small containers (3.5- 13.0 cm^3) (Ersson, 2016) at a density of about 1800- 3500

seedlings per m^2. Sowing is usually done in late summer and seedlings are grown in conventional greenhouses or in climate chambers (where sowing can be done at any date) equipped with artificial light (Mattsson, 2016). The transplants are transferred to cold- (+2°C) or frozen storage (-3°C) when they have obtained the status of being storable, and after 3-4 months in storage they are transplanted into larger containers. So far, methods for determining storability status and post-storage vitality have been developed for conventional 1-1.5-year-old seedlings (Colombo, 1990; Lindström and Håkansson, 1996; L'Hirondelle, 2006). Therefore, in this thesis, one of the objectives was to find out how these methods can be applied to the production of young transplants to improve post-storage vitality (see Study IV).

2 Objectives

The main objectives in the studies were to (Roman numbers indicate particular papers that this thesis is based upon):

I 1) Compare two methods of determining storability: a) shoot freezing tolerance at -25°C evaluated by electrolyte leakage from shoots, and b) shoot dry matter content.
2) Investigate if the shoot electrolyte leakage of frozen stored Scots pine and Norway spruce seedlings can predict post-storage vitality.

II Investigate how the activity of genes related to dormancy and freezing tolerance influence the physiological responses of SD-treated seedlings and if the gene expression profiles could be used to forecast seedling development (i.e., termination of growth, bud set, freezing tolerance, and timing of bud burst).

III 1) Investigate spring-related bud development of seven Douglas-fir provenances, and 2) compare them with the native Norway spruce to evaluate the two species relative to each other, and 3) to study the freezing tolerance of buds at the earliest developmental stages.

IV Investigate how the methods originally developed for conventional 1-1.5-year-old seedlings concerning 1) determination of storability by DMC, the $SEL_{diff\text{-}25}$ method and ColdNSure™, and 2) evaluation of post-storage vitality by SEL measurements can be applied to young late sown seedlings intended for transplanting.

3 Materials and methods

For a more detailed description of the material and methods, see each of the studies I-IV.

3.1 Study locations and seedling material

All seedling material of Norway spruce and Scots pine in the Studies (I-IV) were derived from southern or middle Swedish seed orchards. In the first Study (I) containerized 1-year-old Norway spruce seedlings of two different provenances (59°00'N, alt. 70 m, and 57°00'N, alt. 60 m) and one provenance of Scots pine (60°30'N, alt. 55 m) were grown at Nässja nursery (60°15'N; 16°50'E). The seedlings were delivered in the autumn of 2007 for studies at the research station in Garpenberg (60°15'N; 16°15' E). The second Study (II) was performed in 2013-2014 at the research station in Vassbo (60°31'N; 15°31'E) with container-grown 1.5-year-old seedlings of a Norway spruce provenance (59°30'N, alt. 50 m) cultivated at Nässja nursery (60°15'N; 16°50'E). In the third Study (III) seeds from seven provenances of Douglas-fir originating in British Columbia, Canada, were used in a field trial and a greenhouse study. Four of the provenances were coastal (Caycuse River (48°50'N; 124°29'W, alt. 550 m), Ladysmith (48°57'N; 123°58'W, alt. 549 m), Bella Cola (52°25'N; 126°15'W, alt. 150 m), Bowser Heaman (49°26'N; 124°41'W, alt. unknown)) and three were interior (Three Valley (50°55'N; 118°27'W, alt. 710 m), Anstey Arm (50°58'N; 118°58'W, alt. 610 m), Larch Hills (50°48'N; 119°00'W, alt. 670 m)). In the greenhouse study, conducted in Vassbo research station in 2013-2014, seeds of the seven Douglas-fir provenances together with one Norway spruce provenance (57°00'N, alt. 60 m) were sown on May 13, 2013. For the field trial performed in 2009-2010 near the Asa experimental forest and research station (57°10'N; 14°45'E), located in the southern part of Sweden, 2-year-old seedlings from six out of the seven

Douglas-fir provenances cultivated at Södra's nursery in Falkenberg (56°53'N, 12°32'E) were used (Bowser Heaman was excluded from the field trial). In the fourth Study (IV), performed in 2014-2015 at the research station in Vassbo, the seedling material was young transplants of Norway spruce delivered from the nurseries Flåboda (56°34'N, 15°10'E), Nässja (60°16'N, 16°47'E) and Vibytorp (59°3'N, 15°6'E). The southern Norway spruce provenance (56°40' N, alt. 25 m) was sown on June 22, and sowing of the two middle Swedish provenances (59°00' N, alt. 70 m, and 59°00' N, alt. 125 m) occurred on July 31 and August 1. Seedlings from the three nurseries were frozen stored at four occasions from September to November, 2014. Due to different sowing occasions, the transplants were between 6-11 weeks when the storability tests started.

3.2 Temperature and light conditions

In Study I, sets of previously outdoor stored seedlings delivered from the Nässja nursery were treated differently when they arrived to the research station in Garpenberg. Subsets of seedlings were immediately put into frozen storage (see next section). In order to simulate a warm period in late autumn/early winter, different subsets of seedlings were transferred from open land into a greenhouse on November 23 and kept at +10°C for two months. Thereafter the temperature was raised to +20°C before the seedlings were put into frozen storage (-4°C) on February 4 and February 18, 2008. During the period when the seedlings were exposed to +20°C, the night length was shortened to 8 hours (about the natural night length in early May) by artificial lights. As seedlings were put into frozen storage they were tested for freezing tolerance and storability, see next section.

In the second study, (Study II), seedlings were exposed to short day (SD) treatment (11 hours day, 13 hours night) in a blackout compartment in a greenhouse, starting on July 15, 2013. The lengths of the SD treatments were 0, 7, 14, 21 and 28 days. After termination of each SD treatment, half of each SD-treated batch was transferred outdoors and half remained in the greenhouse, both environments providing natural light conditions. Seedlings in the greenhouse compartment were subjected to higher day and night temperatures compared to seedlings outdoors. On October 28, seedlings stored outdoors were transferred into the greenhouse, and all seedlings were then stored at minimum temperature +7°C and exposed to natural light conditions during winter and spring.

In Study three (III), seedlings intended for the greenhouse trial were grown indoors until June 28, and thereafter transferred to open land, exposed to natural light and temperature conditions until September 25 when they were moved back

indoors. The minimum air temperature in the greenhouse was set to +7°C during winter and spring. The seedlings in the field trial were exposed to natural outdoor conditions. Temperature conditions in the greenhouse as well as in the field trial were recorded by using a data logger (for details see Study III).

In Study four (IV) seedlings grown at three nurseries were sent to the research station in Vassbo at four different occasions during the autumn 2014. Upon arrival the seedlings were put into cold (+3°C) storage and thereafter packed in sealed plastic bags and transferred into frozen (-4°C) storage for different durations, varying between 70-204 days.

3.3 Assessment of dormancy, freezing tolerance, storability, vitality and bud burst timing

During the SD treatment period in Study II, dormancy induction was studied by taking samples each week from July 15 to August 12, and at one final date, August 26, two weeks after termination of SD treatment. Samples were sent to a laboratory for analysis of gene expression profiles of a set of dormancy related genes, previously identified by Stattin et al. (2012). Shoot growth as well as visible apical bud set development were measured each week until August 12, and then biweekly. Bud size for the different treatments in Study II was measured in January, 2014.

Seedling storability was evaluated immediately before the seedlings were put into frozen storage (Study I and IV), as shoot freezing tolerance by using the $SEL_{diff\text{-}25}$ method (Lindström and Håkansson, 1996; Brönnum, 2005). The top 2 cm of the shoots were excised, put in plastic bottles and slowly frozen ($2.5°h^{-1}$) to -25°C. After freezing, deionized water was added and the bottles were put in a shaker for 24 hours. The electric conductivity (EC) in the water, caused by electrolyte leakage (EL) from shoots, was measured with a conductivity meter (Model Hach SensIon 5) before the samples were boiled in an autoclave with the purpose of releasing all ions from the cells (the total EL). The actual effect of freezing was calculated by using the natural electrolyte leakage from unfrozen shoots as a reference. High values (>5) for the difference between EL from injured cells of shoots exposed to -25°C and the natural leakage from cells of unfrozen shoots indicate severe damages caused by freezing. For details about freezing tests and the equations used for calculations, see Study I, III and IV.

Storability was also determined in Study I, II and IV, by measuring dry matter content (DMC) of shoots as described by Rosvall-Åhnebrink (1985). For the DMC test, the upper 2 cm of the shoots were cut off, weighted (fresh) and then dried at 105°C for 24 hours. Thereafter the shoots were reweighted (dry) after

cooling in a desiccator. The DMC was calculated as the ratio between the weights of dry and fresh shoots, in percent (Study I and IV).

In Study II and IV the development of freezing tolerance and storability was also determined by a third method, the commercial molecular test ColdNSure™. For the ColdNSure™ test, the needles were removed from the top 2 cm of the shoots and the apical buds were cut off and grinded in small plastic tubes filled with RNA extraction solution developed by the NSure company. Thereafter a few drops of the liquid were applied on a small paper card and sent to a laboratory for analysis of gene expression profiles of a set of genes associated with freezing tolerance, previously identified by Stattin et al. (2012). Based on gene activity, freezing tolerance was expressed on a scale consisting of four phases (0-3), where the seedlings were classified as storable when they had reached phase 3 (Study II and IV).

In Study I and IV, evaluation of post-storage vitality was performed after frozen storage by regrowth tests (RGC) (Mattsson, 1986). Seedlings were planted in a mix of peat (50% by volume) and sand, and grown for three weeks at an air- and root-temperature equivalent to +20°C, with a photoperiod of 18 hours at a photon flux density of 325 µmol m-1s-1. After three weeks, the newly formed roots were cut off, rinsed in tap water, dried at 105°C for 24 hours and weighted after cooling in a desiccator. Seedlings not showing any signs of new root growth during the test period were classified as dead. The shoot growth after three weeks was measured only in Study I. Post-storage vitality was also evaluated by measuring the natural EL from shoots (SEL) after frozen storage in I and IV. The top 2 cm of the shoots were cut off, and put in plastic bottles with deionized water and then placed in a shaker at 20°C for 24 hours. The SEL (%) was determined by measuring the EC-values before and after autoclaving and then calculate the quotient between these values, multiplied by 100.

In Study III, freezing tolerance of buds of Douglas-fir and Norway spruce, in developmental stage 1, defined as “buds slightly swollen”, and stage 2 defined as “buds swollen” (Krutzsch, 1973), was evaluated by slowly freezing the buds ($2.5°h^{-1}$) down to -5°C, and thereafter measuring the EL. By using samples of unfrozen buds, the actual effect of freezing could be calculated as the difference between EL of frozen and unfrozen buds. For further details see Study III. Also the timing of bud burst in spring was registered for the different provenances of Douglas-fir and the local Norway spruce provenance. In Study II bud burst during spring for the different SD-treated Norway spruce seedlings was followed and compared with untreated control.

3.4 Statistical analyses

In Study II, analysis of variance (ANOVA) was performed on shoot growth data and for differences in bud size. Also, in Study IV, ANOVA was used for testing differences in post-storage SEL (%). In Study I and IV, the chi-square test (Zar, 2010) was used for analysis of differences in survival rates after frozen storage. This test was also used in Study IV for analysis of survival rates after different lengths of frozen storage. In Study II the chi-square test was used to analyse differences in bud set and bud break between seedlings exposed to different SD treatments. In Study III Fisher's exact test (Zar, 2010) was used for statistical analyses of differences in bud development between provenances. Differences in freezing tolerance of buds (Study III) as well as differences in root growth after frozen storage (Study IV) were analyzed using the independent samples t-test with equal variances assumed according to Levene's test for equality of variances (Zar, 2010).

4 Results and discussion

4.1 Freezing tolerance and storability

In order to assess seedling storability three different methods (i.e., freezing seedling shoots to -25°C ($SEL_{diff-25}$ method) (Study I and IV), the molecular test ColdNSure™ (Study II and IV) and determination of DMC in shoots (Study I, II and IV)), were used and tested for their ability to forecast the outcome of long term frozen storage. In Study I, the DMC test did not predict the seedlings storability status as well as the $SEL_{diff-25}$ method. This was especially obvious for seedlings that were exposed to growth promoting temperatures during early winter. When storable seedlings had been exposed to +10°C for two months, both methods gave the same indications i.e. that spruce seedlings still were storable whereas pine seedlings were not. However, when the temperature was raised to +20°C in combination with supplemental artificial light during two additional weeks, the freezing test ($SEL_{diff-25}$ method) gave correct indications that the remaining spruce seedlings in the experiment were not storable anymore. In spite of the fact that all these seedlings were classified as dead after frozen storage the DMC method incorrectly identified them as storable before storage.

All three methods, $SEL_{diff-25}$, DMC and ColdNSure™, were used for assessment of storability of transplants (Study IV). During the first three storage dates (September 16, October 6 and October 27) all methods indicated that none of the three seedling batches were storable, while on the last storage date (November 17) the ColdNSure™ test (Fig. 1) and the $SEL_{diff-25}$ method (Table 1) still gave the same indications for all batches i.e. two batches were considered storable and one batch still not storable. However, for one of those batches that were storable (survival after storage 100%) the DMC values were far below the target level for safe storage. These results are in accordance with findings reported by Stattin et al. (2012) that the molecular test ColdNSure™ and the

$SEL_{diff-25}$ -test are comparable concerning the ability to predict storability. However, both Study I and IV indicate that the DMC method is not as reliable as the other two used methods for assessment of storability. A reason for this could be that the changes of the proportion (%) of dry matter content in shoots is slower than other physiological changes that occur when the seedlings loose or gain freezing tolerance. Obviously this change in seedling status is more effectively measured by electrolyte leakage from shoots after freezing or by analysing the expressions of freezing tolerance related genes by e.g. the ColdNSure™ test. Therefore, under certain circumstances exemplified in I and IV, the DMC method may be unreliable to forecast storability, as earlier shown by Colombo (1990), Dormling (1990) and Lindström (1996).

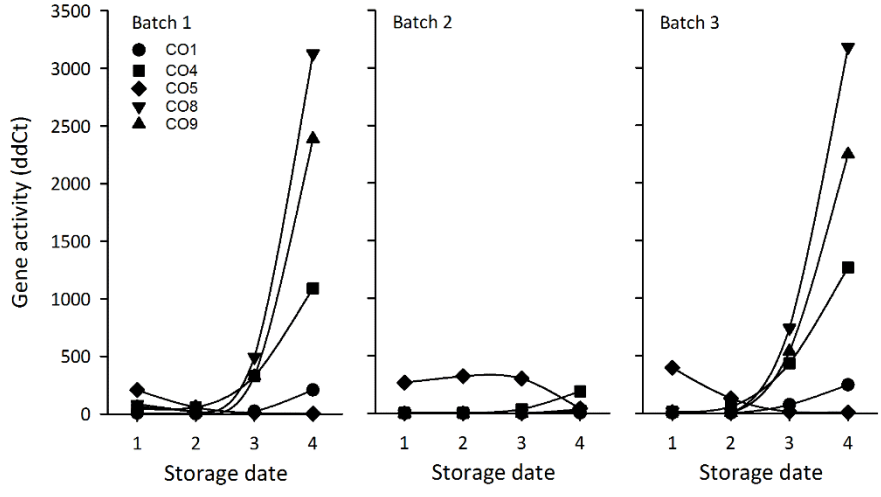

Figure 1. Gene activity profiles of freezing tolerance related genes *CO1*, *CO4*, *CO8*, *CO9* that is known to be upregulated when seedlings become freezing tolerant, and *CO5* which is known to be downregulated (Stattin et al., 2012), for three different batches (1, 2 and 3) measured at four different storage dates (1 = September 16, 2 = October 6, 3 = October 27, and 4 = November 17, 2014). At each sampling date eight apical buds were collected from each batch and analysed for gene activity as a general sample (Study IV).

When applying current target levels for the three methods DMC, $SEL_{diff-25}$, and ColdNSure™, (see e.g. Rosvall – Åhnebrink, 1985; Lindström and Håkansson, 1996; Stattin et al., 2012) for safe long term (5-7 months) storage results from Study IV show that there are good margins for the young seedlings to successfully endure short term (3-4 months) storage before transplanting (Table 1). Considering the shorter storage time for transplants compared to conventional seedlings, results from IV indicate that current target levels could

be modified in order to optimize the time span required to obtain transplants ready for storage.

Table 1. *Storability estimates using the $SEL_{diff-25}$ method (shoot electrolyte leakage following freezing shoots to –25°C subtracted by leakage from unfrozen controls), DMC (%), and the ColdNSure™ method based on the activity of freezing tolerant-related genes at the time of storage. Seedlings of Norway spruce were stored at four different dates (September 16, October 6, October 27, November 17, 2014; length of storage indicated within brackets). For SEL measurements N=4, and for determination of DMC N=4. Standard error within parenthesis. For the ColdNSure™ test, eight apical buds were collected from each batch and each date of storage, and then analysed for gene expressions as one general sample. Survival (%) after storage determined in a 3-week RGC test starting on January 27 and April 9, N=4 (From IV).*

Batch	Storability and survival	September 16 (132/204 days)	October 6 (112/184 days)	October 27 (91/163 days)	November 17 (70/142 days)
1	$SEL_{diff-25}$	16.5 (2.1)	20.2 (1.7)	5.0 (0.9)	0.6 (0.2)
	Storability[1)]	Not storable	Not storable	Not storable	Storable
	DMC	27.7 (3.3)	32.0 (0.7)	33.3 (0.3)	37.6 (0.2)
	Storability[2)]	Not storable	Not storable	Not storable	Storable
	ColdNSure™ [3)]	1	1	2	3
	Surv. (%) Jan[4)]	100	100	100	100
	Surv. (%) Apr[5)]	90	100	90	100
2	$SEL_{diff-25}$	44.7 (3.5)	45.4 (1.7)	47.7 (5.1)	43.7 (11.1)
	Storability[1)]	Not storable	Not storable	Not storable	Not storable
	DMC	22.1 (10.2)	21.0 (0.4)	20.4 (0.4)	23.4 (0.7)
	Storability[2)]	Not storable	Not storable	Not storable	Not storable
	ColdNSure™ [3)]	0	0	0	1
	Surv. (%) Jan[4)]	10	0	30	55
	Surv. (%) Apr[5)]	5	0	0	10
3	$SEL_{diff-25}$	39.2 (0.9)	26.5 (3.6)	12.2 (2.3)	2.5 (1.5)
	Storability[1)]	Not storable	Not storable	Not storable	Storable
	DMC	21.0 (0.5)	24.2 (0.4)	28.9 (0.4)	31.1 (0.3)
	Storability[2)]	Not storable	Not storable	Not storable	Not storable
	ColdNSure™ [3)]	0	1	2	3
	Surv. (%) Jan[4)]	20	20	100	100
	Surv. (%) Apr[5)]	5	0	100	100

[1] *Storability status based on $SEL_{diff\text{-}25}$ threshold values developed for conventional seedlings ($SEL_{diff\text{-}25} \leq 4$)*

[2] *Storability status based on DMC threshold values developed for conventional seedlings (>35%)*

[3] *ColdNSure™ phase based on gene activity measured on conventional 1-year-old seedlings (Phase 0=cold sensitive; Phase 1 and 2=developing freezing tolerance; Phase 3= freezing tolerant and storable)*

[4] *Survival rate (%) after uptake in January*

[5] *Survival rate (%) after uptake in April*

In Study II seedlings exposed to indoor growing conditions generally showed a lower activity of freezing tolerance related genes in autumn compared to seedlings subjected to outdoor conditions which can be seen for the selected gene *CO1* (Fig. 2). These results show the importance of low temperature exposure for the hardening processes during autumn and that possibly warmer autumn climate can interfere with a normal development of freezing tolerance. The fact that both temperature and photoperiod are important factors affecting dormancy induction and the development of freezing tolerance has earlier been reported by for example Dormling et al. (1968), Weiser (1970), Heide (1974a), Christersson (1978), as well as Flöistad and Granhus (2010).

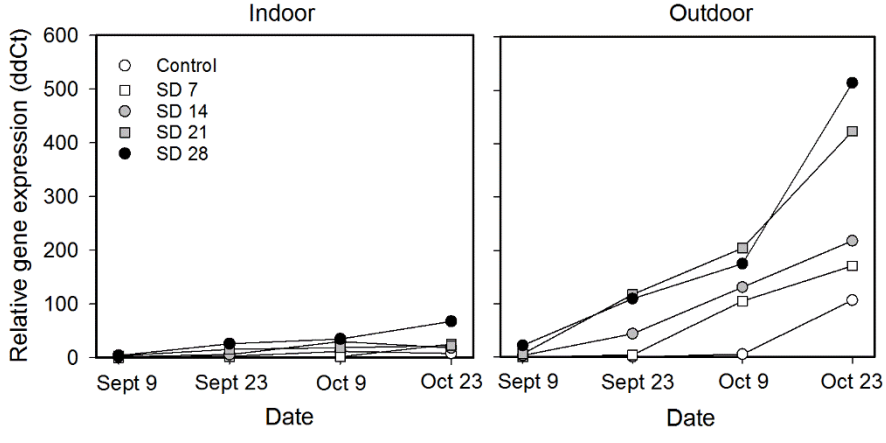

Figure 2. Expression profile of the gene *CO1* used to indicate freezing tolerance. The level of gene activity is expressed as ddCt (delta delta threshold cycle), which is the relative difference between the gene of interest and reference genes (genes that did not show any significant difference in expression). Measurements were performed at four different occasions, September 9, September 23, October 9, and October 23, 2013, for Norway spruce seedlings previously subjected to SD (short

day) treatments of various lengths with a photoperiod of 11 hours (0, 7, 14, 21 and 28 days) starting on July 15. After SD treatment seedlings were either kept indoors or moved outdoors. Each sampling day, eight apical buds were collected from each SD treatment and growing environment and then analysed for gene expression as one general sample (Study II).

In Study II, when comparing the outcome of the ColdNSure™ test and the DMC values on October 23, results showed that both methods indicated that indoor stored seedlings were not storable while outdoor stored seedlings had reached the target levels for safe storage. The molecular test ColdNSure™ indicated a very low activity, far from the target level, of freezing tolerance-related genes for the indoor stored seedlings. The difference between DMC values for indoor and outdoor stored seedlings were small which supports the conclusion by Colombo (1990) and Lindström (1996) that small changes in dry matter content result in large differences in storability making the method unprecise (Fig. 3).

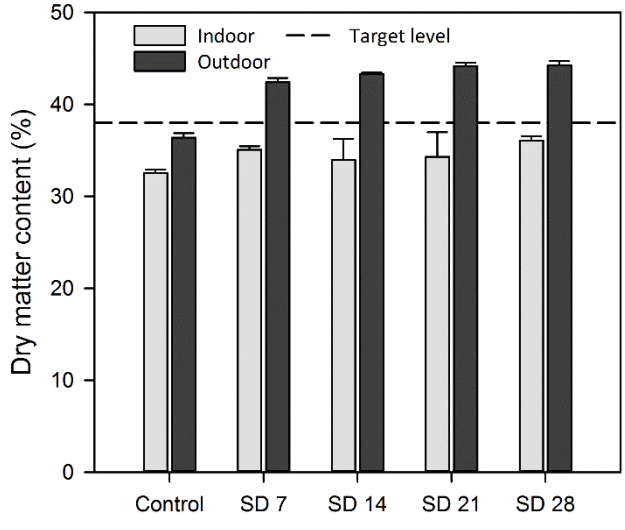

Figure 3. Dry matter content (DMC %) for control seedlings and SD (short day) treated (11 h light; 13 h night) seedlings for 7, 14, 21, and 28 days, grown indoors and outdoors measured on October 23, 2013. According to Rosvall-Åhnebrink (1985) the target level for storability of SD-treated Norway spruce seedlings is ≥38% (dashed line). DMC (%)-values based on shoots from 20 seedlings, N=4. Vertical lines represent the standard error (Study II).

Results from Study I and II suggest that warmer autumns could cause problems with the winter hardening processes of seedlings. The time point when seedlings are ready for long-term freezer storage can be delayed due to insufficient development of freezing tolerance and autumn planting may become critical due

to risk of increased sensitivity to late-season frost. As also stated by Bigras et al. (2001) warmer autumn temperatures may postpone the development of root freezing tolerance, which increase risks for overwintering damage.

4.2 Expression profiles of dormancy related genes as a response to SD treatment

The impact of SD treatment has previously been based on measurements of changes in growth, bud status and freezing tolerance. Study II indicated that 7 days of SD treatment is as effective as longer SD treatments to terminate apical shoot growth, which is in accordance with several other studies also testing growth effects of short periods of SD treatment on spruce (see e.g. Dormling et al., 1968; Heide, 1974a; Konttinen et al., 2003; Flöistad and Granhus, 2013).

The expression profiles of the selected dormancy related genes *LN2*, *LN3* and *LN4* (Fig. 4), earlier identified by Stattin et al. (2011; 2012), indicated that spruce seedlings need at least 14 days of SD treatment (photoperiod 11 h), for dormancy induction. In Study II, as well as in Stattin et al. (2011), seedlings exposed to the longer (21-28 days) SD treatments showed a higher gene activity compared to seedlings from shorter (7-14 days) durations. Seedlings subjected to outdoor conditions after termination of SD treatment showed an earlier activation of dormancy related genes, as well as earlier expressions of freezing tolerance related genes, compared to seedlings exposed to indoor conditions. Our results are supported by previous studies by Rosvall-Åhnebrink (1982), Konttinen et al. (2003) and Kohmann and Johnsen (2007), showing that longer durations of SD treatments promote the development of freezing tolerance. High activity of dormancy related genes indicated a deeper state of dormancy, which in Study II also resulted in a reduced risk for a second bud flush in autumn. Flöistad and Granhus (2013) also reported an increased risk for a second bud flush among seedlings after only 7-14 days of SD treatment, especially if the SD treatment started early (in late June). As shown in Study II, the increased risk for a second bud flush after none or short durations of SD treatments can be due to the limited stimuli of dormancy related genes during the first weeks of treatment (Fig.4).

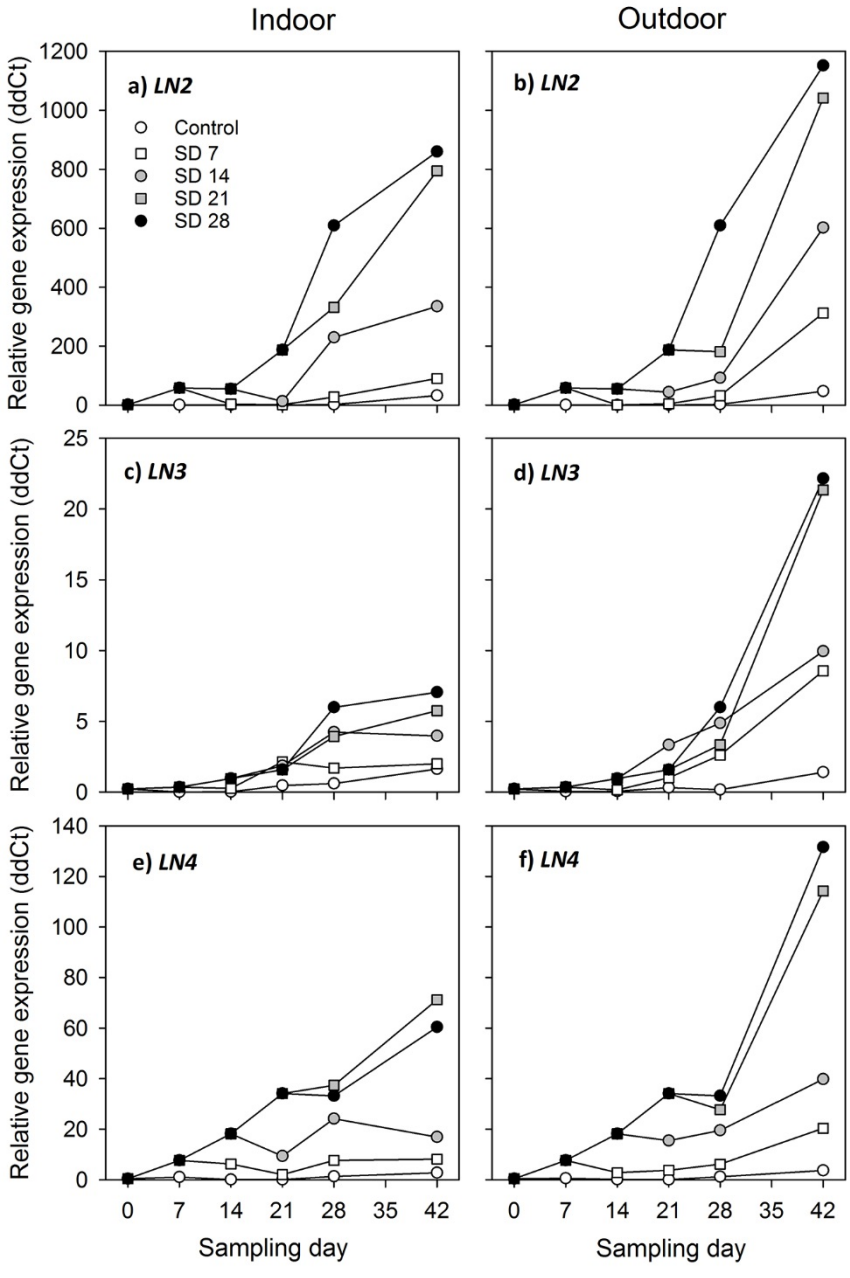

Figure 4. Gene expression profiles of *LN2* (a, b), *LN3* (c, d) and *LN4* (e, f) used for indicating dormancy induction. The level of gene expression is given as ddCt (delta delta threshold cycle), which is the relative difference between the gene of interest and reference genes (genes that did not show any significant difference in expression). SD (short day) treatments (7, 14, 21 and 28 days) with a photoperiod of 11 hours started on July 15. Measurements were performed on July 15 (day 0), July 22 (day 7), July 29 (day 14), August 5 (day 21) and August 12 (day 28) and on

August 26 (day 42). After termination of SD treatment, the Norway spruce seedlings were either kept indoors (a, c, e) or moved outdoors (b, d, f). Each sampling day, eight apical buds were collected from each SD treatment and growing environment and then analysed for gene expression as one general sample (Study II).

An additional minor study (data not published) was conducted to investigate how many days of SD treatment were needed to provoke terminal shoot growth cessation of the Norway spruce provenance used in Study II. The study started in the middle of July 2015. Treatments consisted of none (0 days) or short (3, 6, 9 and 12 days) SD treatment with measurements of apical shoot growth every third day for 15 days, followed by weekly measurements thereafter. SD treatment corresponding to a photoperiod of 11 h was performed indoors at a mean temperature of approximately +25°C, and after termination of SD treatment the seedlings were moved outdoors. Compared to the untreated control seedlings that continued shoot elongation until late August, the results showed that 3 days of SD treatment reduced apical shoot growth, but the final termination of shoot growth seems to have occurred approximately at the same time for these two treatments. SD treatment during 6 days terminated shoot growth much earlier than 3 days, but 9 and 12 days of SD treatment were most effective in shoot growth cessation (Fig. 5). A previous study by Heide (1974a) showed that 1-2 days of SD treatment with a photoperiod of 10 hours at air temperatures +18-24°C, was sufficient to reduce apical shoot growth, and 4 days of SD treatment resulted in a temporary termination of shoot growth. However, seedlings from all short (1, 2 and 4 days) SD treatments resumed growth when transferred back to natural light conditions while the seedlings from the longer (8 and 16 days) durations reached a stable growth termination. It would be interesting to follow how the activity pattern of dormancy related genes develop over time after these very short durations of SD treatment to compare with results obtained in Study II. In Study II, 7 days was enough to effectively shut down apical shoot growth. However, the activity of our selected dormancy related genes after 7 days of SD treatment was expressed to a much lesser extent and showed a delayed response, compared to the longer SD durations. This indicates that there may be other sets of genes that could better reflect growth responses of different SD treatments. Searching for other sets of genes that are more sensitive in reflecting seedling reactions during dormancy induction is a task for further research. However, as previous studies (Stattin et al., 2012) have showed strong correlations between gene activity and freezing tolerance there is less need for exploring new sets of genes explaining freezing tolerance and storability.

As the effect of different lengths of SD treatment on gene activity only appears after a few weeks (Fig. 4) a practical way for nurseries to decide when termination of SD treatment can be done is to measure shoot growth daily inside

the blackout compartment. Results obtained in Study II indicate that when apical shoot growth has terminated, a continued SD treatment for about 1-2 weeks could be enough to ensure that the dormancy related genes have been activated so that true dormancy is obtained. Further research is needed, however, for determination of the time span between the termination of apical shoot growth and the initiation of dormancy.

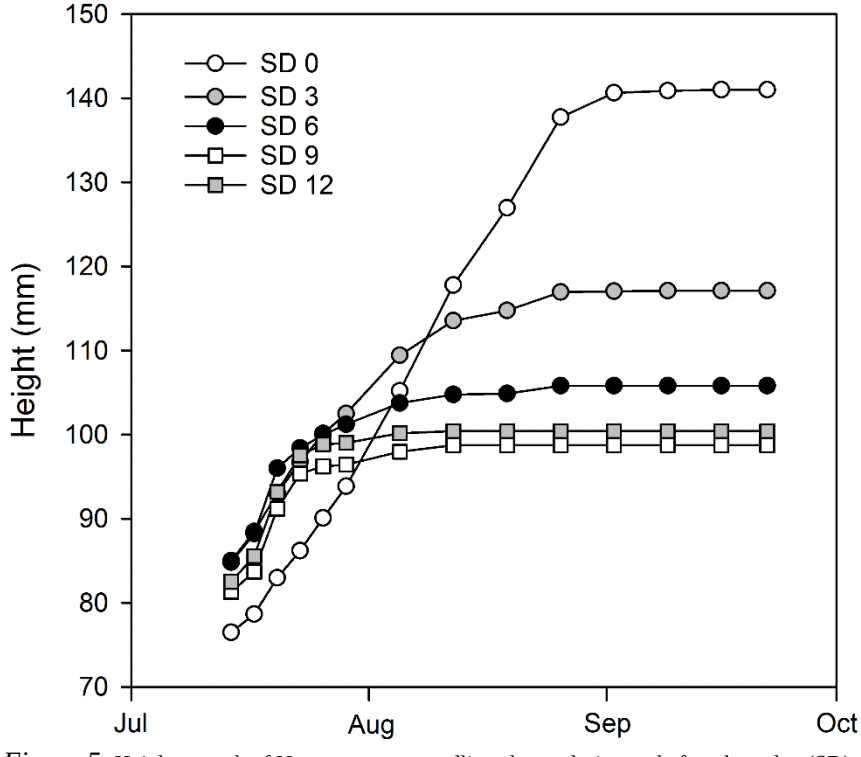

Figure 5. Height growth of Norway spruce seedling shoots during and after short-day (SD) treatments corresponding to a photoperiod of 11 hours. The SD treatments (0, 3, 6, 9 or 12 days) were done in an indoor blackout compartment, started on July 14, 2015, and the seedlings were transferred outdoors after termination of each SD treatment. Height measurements were performed every third day for the first 15 days starting on July 14, thereafter biweekly from August 5 to September 22. Total number of seedlings from each treatment = 44, N=4 (each replicate contains 11 seedlings) (exclusively published for this thesis).

4.3 Post-storage vitality

Results from Study I with 1-year-old spruce and pine seedlings, and young late sown transplants of spruce in Study IV showed that measuring the natural leakage from shoots after frozen storage can be used for assessment of post-

storage vitality. Correlations were found between SEL (%) and mortality (Fig. 6 and Fig. 7) as well as between SEL (%) and the capability for root growth after planting for both conventional seedlings (Study I) and young transplants (Study IV). Depending on when seedlings were put into storage, the duration in storage for the older seedlings in Study I was 1.5-6 months, while the young transplants were stored for either 3-4 months, or 5-7 months before they were tested for vitality.

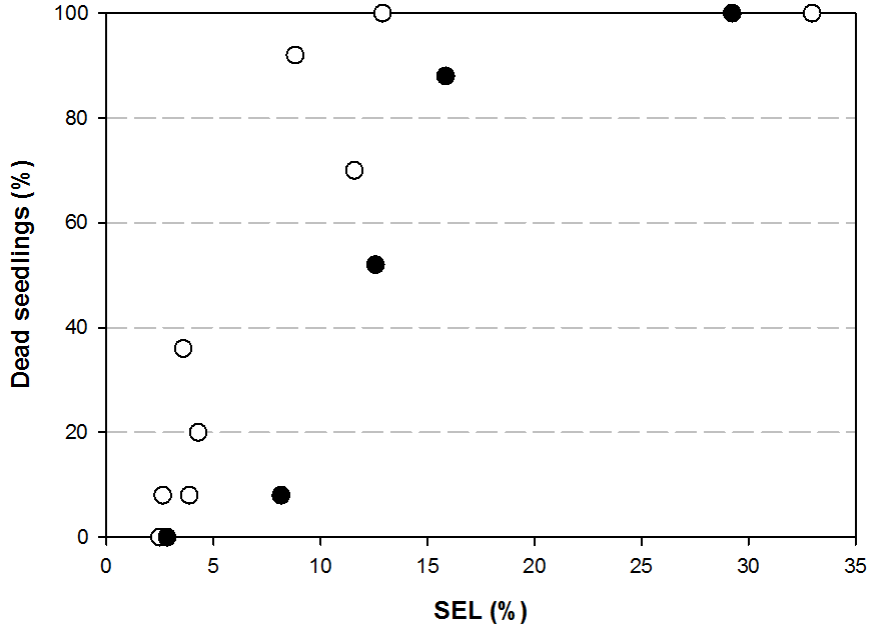

Figure 6. The relation between shoot electrolyte leakage (SEL%) and seedling mortality (%) within each investigated batch consisting of either Scots pine (●) or Norway spruce (○) seedlings subjected to frozen storage. Storage started at six different dates (September 27, October 1, October 8, October 15, February 4, and February 18) during autumn and winter 2007/2008. Shoot electrolyte leakage (SEL %) was measured on 15 seedlings in 5 replicates from each batch just before planting seedlings in a growth test at the end of March. Post-storage survival, determined 3 weeks after planting was based on 20–25 seedlings in 4–5 replicates/batch. Logarithmic regressions resulted in $R^2 = 0.826$ for Scots pine and $R^2 = 0.831$ for Norway spruce (Study I).

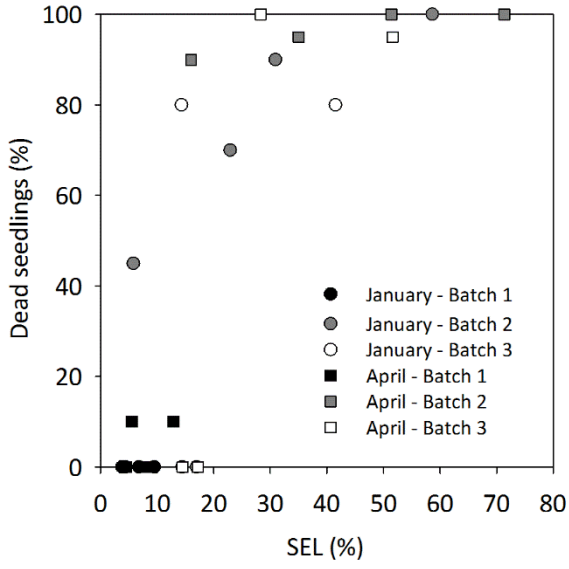

Figure 7. The relation between shoot electrolyte leakage (SEL %) and seedling mortality (%) for each of three batches (1-3) measured after frozen storage. The three different batches were frozen stored at four different dates (Table 1) and stored until January or April. Shoot electrolyte leakage (SEL %) was measured on 20 seedlings (5 seedlings in 4 replicates) from each batch before planting seedlings in a regrowth test (RGC) starting on January 27 and April 9, respectively. Post-storage mortality (%) determined 3 weeks after planting was based on 20 seedlings (5 seedlings in 4 replicates) for each batch and storage date. For the relation between SEL (%) and mortality (%) logarithmic regressions for all observations resulted in R^2=0.6354 (Study IV).

For both Study I and IV, seedlings with a SEL value <5% had the highest (88-100%) survival rates after storage. A SEL value of 5-10% resulted in low (50%) survival for the older seedlings (Study I) while the young transplants (Study IV) within the same SEL interval still showed high (85-90%) survival rates. The common trend was that the young transplants showed a better survival and regrowth at higher SEL values compared to older, conventional seedlings. A reason for this can be differences in cell structures between young and older seedlings resulting in higher natural leakage levels from shoots of young seedlings.

Generally, mean values of post-storage SEL increased when the duration of storage was extended from January to April (Study IV), especially for seedlings from the early autumn storage dates. This indicates that the vitality of young transplants is negatively affected by a longer storage time. The RGC test also showed that survival decreased and the ability to produce new roots was reduced when time in frozen storage increased (Fig. 8). This is in accordance with Stattin et al. (2012) who reported that longer periods of frozen storage resulted in lower vitality of seedlings. Ritchie (1982) showed that seedlings' carbohydrate

reserves are known to gradually deplete during cold storage which could be a reason for the decline in root (and shoot) growth after the longer duration in storage. In addition to this, young transplants have smaller carbohydrate reserves compared to conventional seedlings which could strengthen this effect. Lindström and Stattin (2010b) hypothesized that the limited amount of carbohydrates stored in young (9-15 weeks) Norway spruce and Scots pine seedlings were partly spent through respiration during frozen storage, resulting in low post-storage vitality.

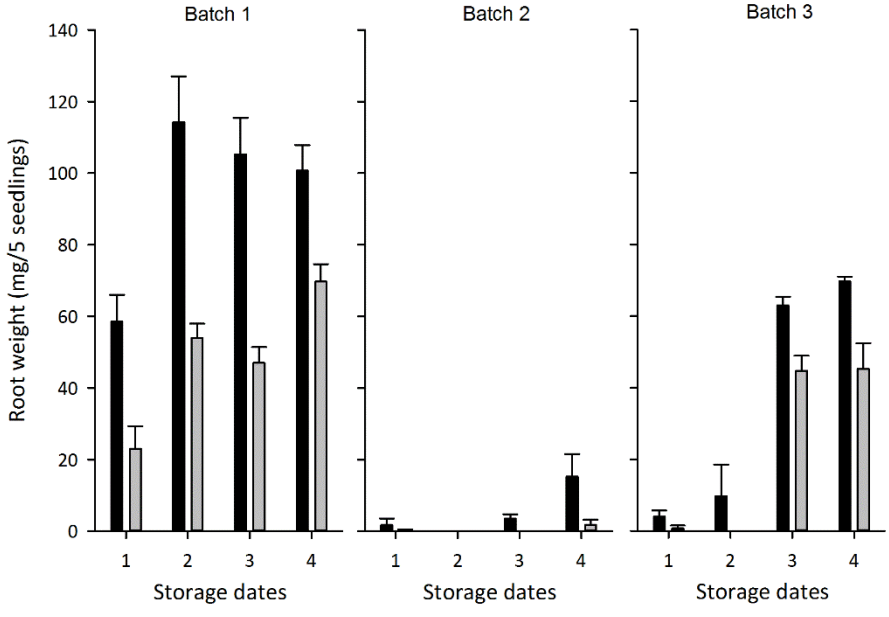

Figure 8. Post-storage vitality measured as root growth (dry weight mg/5 seedlings) in 3-week regrowth tests starting on January 27 (black bars) and April 9 (grey bars) for batch 1, batch 2 and batch 3. Measurements based on 20 Norway spruce seedlings (5 seedlings from 4 replicates) for each batch and storage date (1 = September 16, 2 = October 6, 3 = October 27, 4 = November 17, 2014). Vertical lines represent the standard error (Study IV).

A conclusion from Study I and IV is that the SEL method could probably be used as a "screening test" to identify batches with reduced vitality. The SEL method is relatively fast and easy to perform, and only simple laboratory equipment is needed. If SEL values are high the next step in a practical situation is to perform an RGC test (Mattsson, 1986) to further investigate the vitality of the seedlings. An advantage with the RGC test is that it makes it possible to detect damages to roots. It is unclear if measurement of leakage from shoots can give any indication of the root status. Further research is needed to investigate such possible correlations and to develop a complete kit of methods that also

includes the vitality of roots as roots are easily damaged by low temperature exposure (see e.g. Lindström and Nyström, 1987; Lindström and Stattin, 1994; Bigras et al., 2001)

4.4 Bud burst in spring

Study II also included observations on how different lengths of SD treatment affect the timing of bud burst in spring. The majority of seedlings subjected to the longer durations of SD treatment (21 or 28 days) with starting date July 15, showed a somewhat earlier bud burst the following spring compared to seedlings exposed to the shortest SD treatment (7 days)(Fig. 9). Similar results have earlier been reported by for example Konttinen et al. (2003) and Flöistad and Granhus (2010). Luoranen and Sutinen (2017) found that SD treatment of Norway spruce seedlings led to fewer and shorter protective bud scales, and an early loss of bud scales compared to untreated control seedlings, which may cause bud damage due to spring frosts. These findings are in accordance with the results from Study II showing that the longer SD treatments resulted in smaller buds, probably due to lack of photosynthetic light. This could be an argument that it is important not to exaggerate the length of dark periods during SD treatment.

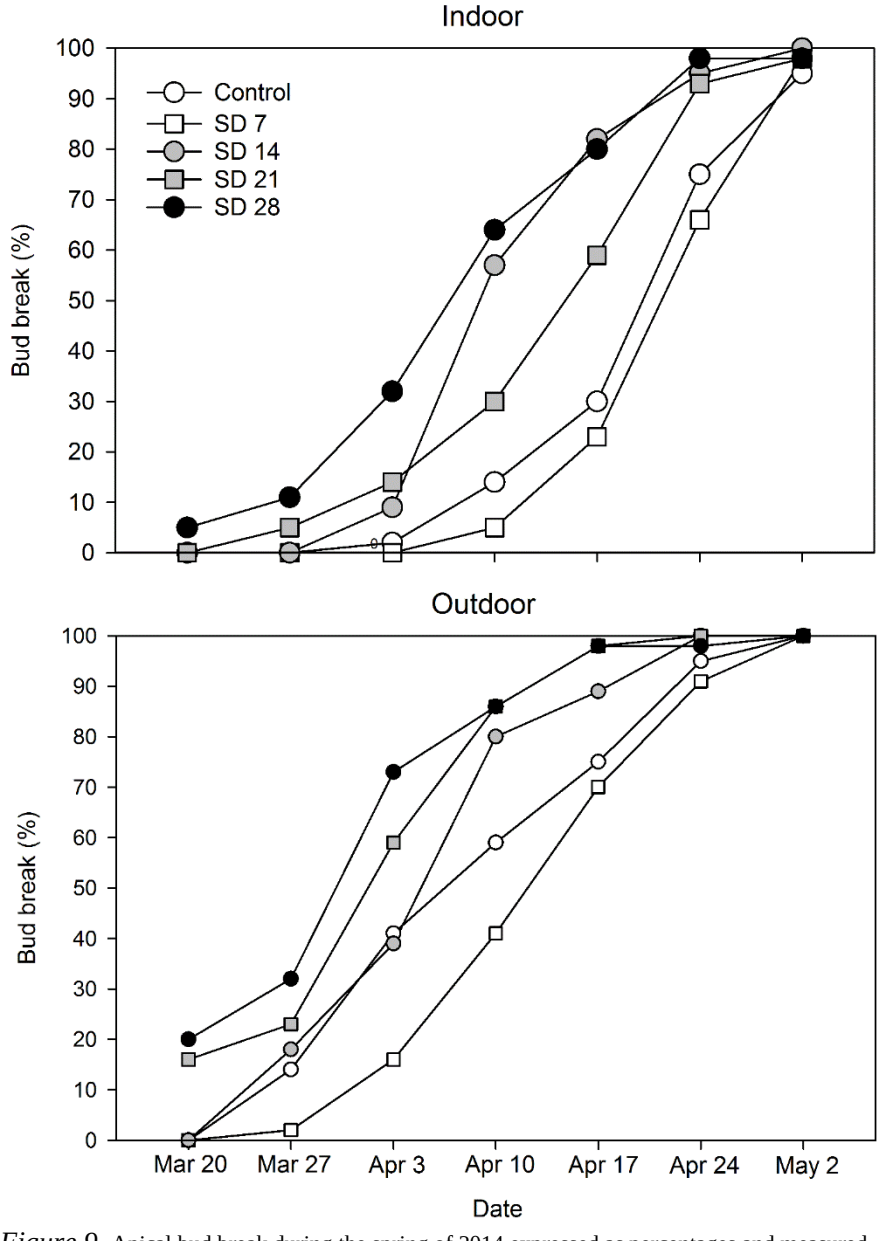

Figure 9. Apical bud break during the spring of 2014 expressed as percentages and measured weekly between March 20 and May 2. Seedlings had been subjected to short-day (SD) treatments (11 h light; 13 h night) of various lengths starting on July 15, 2013 and thereafter kept indoors or outdoors. Control seedlings in both growing environments were kept under natural day lengths. Seedlings previously grown and stored in outdoor conditions were transferred indoors on October 28, 2013 (Study II).

As stated in Study II there are large differences in activity of dormancy related genes between seedlings from longer and shorter SD treatments (Fig. 4). The well-known fact that longer durations of SD treatment result in earlier bud burst leads to the conclusion that it could be possible to use dormancy gene expression profiles not only to predict the forthcoming development of seedlings in autumn, but also the timing of bud burst in spring. A tool based on such gene measurements could simplify the selection of SD treatment programs. The tool could also be used to facilitate the selection of suitable provenances for spring planting on locations with high risks for frost.

Knowledge concerning the timing of bud set in autumn as well as bud burst in spring is important for selecting suitable provenances of Norway spruce, and also for Douglas-fir. Previous studies by Howe et al. (2003) and Sögaard et al. (2007) showed strong correlations between spring frost hardiness and bud burst occurrence, which makes bud burst occurrence an important indicator of species suitability for a particular planting location (Hannerz, 1999; Howe et al., 2003; Anekonda et al., 2004; Gould et al., 2011). Douglas-fir seedlings are known to be sensitive to spring frost due to early bud burst (Malmqvist, 2017). In Study III interior provenances of Douglas-fir showed an earlier bud burst compared to coastal provenances (Fig. 10), and these results are in accordance with previous studies by for example Campbell and Sugano (1979) and Edman (1997). Previous provenance trials performed in Norway, Sweden and Finland indicated that interior provenances of Douglas-fir were more suitable for planting in Nordic conditions compared to coastal provenances (Kurkela, 1981; Magnesen, 1987). In spite of these provenances being more exposed to damage by early spring frost than coastal provenances, they showed better field performance. A reason for this is probably an adaptation to a more continental climate resulting in a more rapid development of freezing tolerance in the autumn among interior provenances of Douglas-fir compared with coastal provenances. This reduce risks for severe autumn frost injuries and winter desiccation (Malmqvist et al., 2016).

The local provenance of Norway spruce used in Study III showed a similar timing of bud burst as the interior Douglas-fir provenance Three Valley. This result together with results from freezing tolerance tests in autumn showing that the Three Valley provenance had a similar pattern of hardening as the local Norway spruce (Malmqvist et al., 2016) led to the conclusion that the Three Valley provenance could be used for planting in the zones recommended for the provenance Öhn (lat. 57°00’N) of Norway spruce used in Study III.

The results in Study III also showed that buds of Norway spruce and Douglas-fir were sensitive to sub-zero temperatures (-5°C) already in their earliest stage of bud burst (i.e. stage 1; buds slightly swollen) according to the

Krutzsch index (Krutzsch, 1973). The risk for spring frost damages during the first year in the field can, however, be reduced by planting cold- or frozen stored seedlings late in spring or early summer.

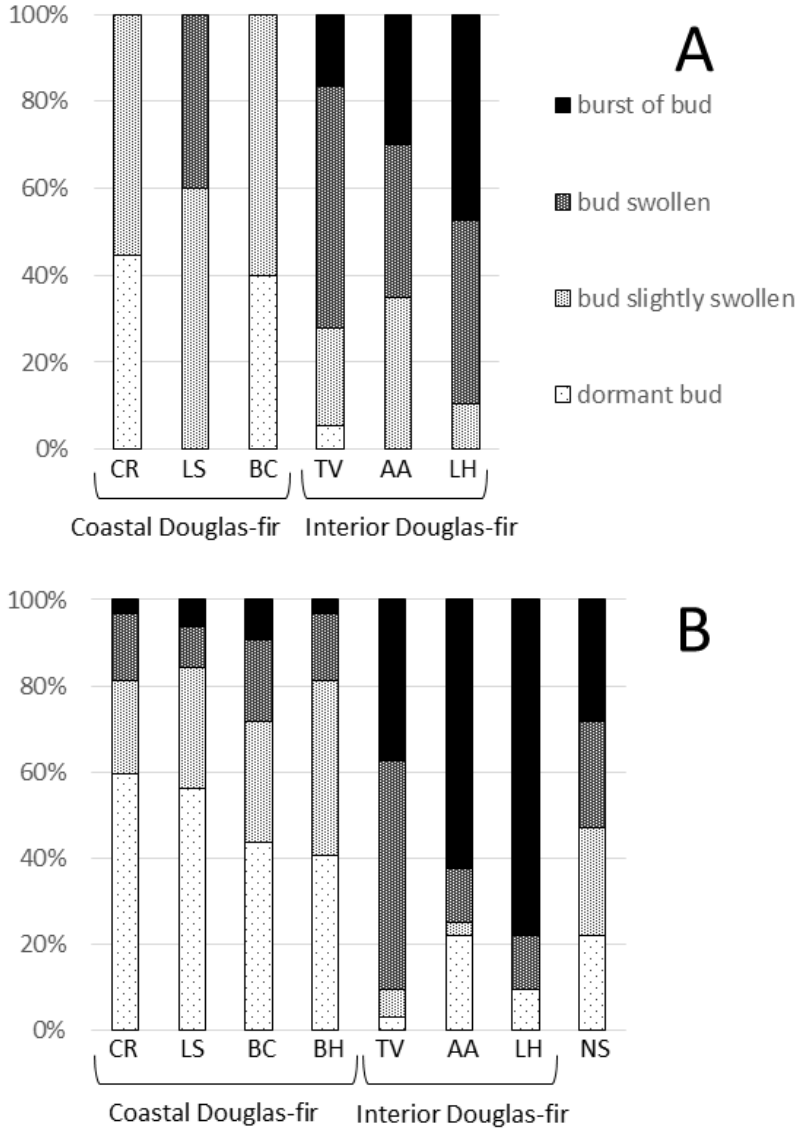

Figure 10. Percentages of field-grown (A) and greenhouse-grown (B) seedlings in different spring-related bud development stages, using the Krutzsch index (1973) for the seven provenances of Douglas-fir (CR=Caycuse River; LS=Ladysmith; BC=Bella Coola; BH=Bowser Heaman; TV=Three Valley; AA=Anstey Arm; LH=Larch Hills) and one provenance of Norway spruce (NS).

Selected data showing bud burst status in field on May 19, 2010 (A), and from the greenhouse experiment on April 1, 2014 (B). N=32 (redrawn from Study III).

As the greenhouse study and the field trial of Study III showed the same pattern concerning bud burst development of different provenances of Douglas-fir (Fig. 10), it indicated that greenhouse studies could supplement and in some cases, replace field studies. This could facilitate the selection of suitable provenances for planting. Also, for Douglas-fir as for Norway spruce, it could be possible to use expression profiles of dormancy related genes to forecast seedling development in autumn and in spring. However, to accomplish this, investigations are needed to identify suitable sets of genes related to dormancy induction for Douglas-fir.

Jacobs et al. (2008) showed that SD treatment of Douglas-fir effectively improved the development of autumn freezing tolerance. As the Douglas-fir provenances derive from latitudes normally far south of the planting locations in Sweden, the hardening processes are delayed resulting in high risk for frost damages in autumn (Malmqvist et al., 2016). As well as for spruce, SD treatment could be an option in Swedish nursery practice to ensure that Douglas-fir seedlings get ready for frozen storage at an early date and can be autumn planted without suffering from frost. Still, there is a need for further studies investigating the combined short- and long term effects of using different SD treatments and provenances of Douglas-fir.

5 Conclusions

- Among the three tested methods to assess storability of seedlings, measurements of DMC in shoots were not as reliable to predict the outcome of storage as determination of low temperature (-25°C) tolerance in shoots (the $SEL_{diff-25}$ - method) or the molecular test ColdNSure™.
- Young late sown transplants can be successfully short term stored before reaching the target levels for safe storage of the DMC, $SEL_{diff-25}$ and ColdNSure™ method. This indicates that current target levels for safe storage could be modified to better suite this kind of seedling assortment.
- A longer period (21-28 days) of SD treatment with a photoperiod of 11 hours resulted in more rapid activation of selected dormancy related genes and an earlier development of freezing tolerance, compared to none and shorter (7-14 days) SD treatments.
- SD treatment for 28 days resulted in smaller, visible buds compared with the shorter (7-14 days) treatments, probably due to lack of photosynthetic light during bud set.
- Expression profiles of selected dormancy related genes can be used as an indicator of seedling status in terms of dormancy induction. The profiles could also possibly be used to predict development of freezing tolerance, bud set, risks for a second bud flush in autumn and the timing of bud burst in spring.
- Gene expression profiles for Norway spruce show that besides photoperiod the temperature during autumn plays an important role for the dormancy induction and the development of freezing tolerance. Norway spruce seedlings will reach a storable state within a reasonable time if they are SD-treated in combination with normal outdoor climate conditions. Warm temperatures during autumn may severely delay the hardening processes.
- Freezing tolerant and storable spruce and pine seedlings of about the same origin that were exposed to a warm period lasting for about 2.5 months during

mid-winter showed a rather slow dehardening. By the end of the period however, the pine seedlings were the first to become unstorable.

- Seedling post-storage vitality can accurately be determined by measuring the natural electrolyte leakage from shoots (the SEL-method) of pine and spruce seedlings. Correlations were obtained between SEL (%) and mortality as well as between SEL (%) and root growth for both conventional 1-1.5-year-old spruce and pine seedlings as well as for young, late sown transplants of Norway spruce. The SEL-method has the potential to serve as a first "screening test" to identify batches with deteriorated vitality for further investigations.
- A prolonged storage time of young transplants resulted in lower survival as well as lower root growth capacity and higher levels of SEL, especially for seedlings stored at earlier dates (in September).
- Results from a greenhouse experiment showed that the interior Douglas-fir provenance Three Valley had a similar timing of bud burst in spring as the local Norway spruce. This result in combination with previous findings that Three Valley was found to be freezing tolerant and storable at an early date in autumn, likely makes this provenance suitable for planting in Swedish conditions in the same recommended areas as the local provenance of Norway spruce (lat. 57°00'N, alt. 60 m).
- Results from a field trial and a greenhouse study showed similar results concerning bud burst patterns of a number of Douglas-fir provenances. This indicates that greenhouse studies can be used as a complement to field trials.
- Buds of Norway spruce and Douglas-fir were found to be sensitive to sub-zero temperatures already in their earliest developmental stage (stage 1=buds slightly swollen) according to the Krutzsch index (1973).
- My thesis points out that there still are much research to be done concerning development of practical tools in the nurseries to get better control of seedling quality. Further studies are needed to identify additional sets of genes that can be related to different kinds of seedling reactions. This could be obtained by comparing the physiological outcome of different nursery treatments with expressions profiles of different sets of genes, thus continuing the work that has been presented in this thesis. In a global perspective the results from this work emphasizes the sensitivity of conifer seedlings to changes in temperature climate. Warm autumns as well as warm periods in winter may have an inhibitory impact on the normal hardening and dehardening processes of young trees.

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Popular science summary

In Sweden, 350-400 million forest seedlings are produced annually for planting, and about one third of these are put into frozen storage in late autumn for winter storage which makes it important to investigate if the seedlings are ready for frozen storage. Storability assessment of 1-1.5-year-old Norway spruce (*Picea abies* (L.) Karst.) and Scots pine (*Pinus sylvestris* (L.)) seedlings intended for long term (5-7 months) frozen storage was performed using three different methods. Measurements of dry matter content in shoots were found not as reliable as determination of low temperature (-25°C) tolerance of shoots (the $SEL_{diff\text{-}25}$ method) or by determining the activity level of genes associated with freezing tolerance by using the molecular test ColdNSure™. These three methods were also used for storability assessment of young, late sown spruce seedlings intended for transplanting. This assortment has become more common in Swedish forest tree nurseries and has the advantage of providing a better utilization of cultivation and storage areas. The results indicate that young transplants became storable before reaching the target levels for safe long term storage of conventional 1-1.5-year-old seedlings. This means that current target levels for safe storage should be modified if the methods are to be used with young seedlings intended for short term (3-4 months) freezer storage.

Post-storage vitality can easily and rapidly be determined by measuring the natural electrolyte leakage from shoots (SEL) for older (1-1.5-year-old) pine and spruce seedlings as well as for young transplants. Measurements of SEL as well as results from regrowth tests showed that the vitality of young transplants decreased when the time in storage was prolonged from 3-4 months up to 5-7 months.

To investigate how already storable and freezing tolerant seedlings react to a warm period during late autumn or early winter, outdoor stored seedlings were transferred into a warm greenhouse (+10-20°C) and kept there for two and a half months. The results showed that the seedlings kept their freezing tolerance for a

long time (at least two months), and that of two comparable origins of pine and spruce, the pine seedlings lost freezing tolerance somewhat earlier than spruce.

At forest tree nurseries in Scandinavia it is common practice to perform short-day (SD) treatment of spruce seedlings during late summer. Short days are achieved by using black curtains that completely covers the seedlings for about 11-13 hours a day. The purpose with the SD treatment is to make the seedlings terminate shoot growth and start to prepare for low winter temperatures. SD treatment in combination with low outdoor temperatures during autumn was efficient to make the seedlings storable at an earlier date. By using molecular tests and thereafter analyse the activity level of dormancy related genes and genes associated with the development of freezing tolerance, the effects of different SD treatments as well as other environmental changes can be determined. The results showed that even a short (7 days) period of SD treatment resulted in increased activity among genes related to both dormancy and the development of freezing tolerance when compared to gene activity of untreated control seedlings. Seedlings exposed to longer (21-28 days) SD treatments entered a deep dormancy phase and were classified as freezing tolerant and storable at an earlier date compared to seedlings from the shorter (7-14 days) SD treatments. Gene expressions mirror the seedling status too late to be used as a tool for decision support regarding when to end SD treatment. Instead, termination of shoot growth could be used to decide the point in time when SD treatment can be ended. If necessary, two additionally weeks of SD treatment would ensure that deep dormancy will be obtained. However, gene expression profiles have the potential to be used for assessment of seedling dormancy status, predicting timing of bud set and development of freezing tolerance. Analysis of gene activity could also forecast the risk for a second bud flush in autumn as well as the timing of bud burst in spring.

The interest in Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) has increased in Sweden, but the availability of seedlings of suitable provenances has been limited. Seedlings of Douglas-fir are known to be very sensitive to frost damages, which makes timing of bud burst in spring as well as timing of autumn freezing tolerance development important factors when selecting suitable seedlings for planting. To determine differences in bud burst timing of a few selected provenances of Douglas-fir, a field trial and a greenhouse study were performed. In the greenhouse trial, also a local provenance of Norway spruce was included. The field trial and the greenhouse study showed the same pattern concerning time of bud burst for a number of Douglas-fir provenances, indicating that greenhouse screening tests can be used for provenance selection. Earlier studies of the winter hardening process of the interior provenance Three Valley have shown similar development as for a local provenance of Norway

spruce (lat. 57°00’N). In this study bud burst patterns were similar for Three Valley and the local spruce. This indicates that among the Douglas-fir seedlings tested, the interior provenance Three Valley would have a good chance of a successful establishment in southern Sweden.

Populärvetenskaplig sammanfattning

I Sverige produceras årligen 350-400 miljoner skogsplantor och cirka en tredjedel av dessa övervintras i fryslager. Unga omskolningsplantor av gran (*Picea abies* (L.) Karst.) ämnade för kort fryslagring visade sig vara lagringsbara innan de uppnått de gränsvärden för säker långtidslagring som utvecklats föräldre konventionella plantor med avseende på torrsubstanshalt, frystolerans och det molekylära testet ColdNSure™. Resultaten tyder också på att mätningar av torrsubstanshalt kan vara missvisande, inte bara för gran utan även för tall (*Pinus sylvestris* L.).

Plantvitaliteten efter lagring kan enkelt och snabbt fastställas genom att mäta det elektrolytiska läckaget från skotten (SEL) hos äldre (1-1,5-åriga) plantor samt unga omskolningsplantor. Vitaliteten hos omskolningsplantorna sjönk enligt SEL-mätningar och i termer av överlevnad och tillväxt i odlingstest om fryslagringen förlängdes från 3-4 upp till 5-7 månader. För att testa hur tall- och granplantor reagerar på en varm period under sen höst- tidig vinter placerades utomhuslagrade, redan invintrade plantor i ett varmt växthus (+10-20°C) under två och en halv månad. Resultaten visade att plantorna behöll sin köldhärdighet länge (cirka 2 månader), men att tallen förlorade sin köldhärdighet något snabbare än granen.

Granen avslutar sin skottsträckning redan efter 7 dagar om man mitt i sommaren förlänger natten till ca 11-13 timmar med hjälp av utrustning för mörkläggning. För att ta reda på hur många veckor som plantorna behöver mörkläggas för att plantorna ska uppnå en djup vila och bli lagringsbara, studerades aktiviteten hos de gener som styr plantornas vila och utveckling av frystolerans. Resultaten för 1-åriga granplantor som långnatts(LN)-behandlades under 0, 7, 14, 21 och 28 dagar visade att 7 dagars LN-behandling är lika effektiv som längre behandlingar (21-28 dagar) för att granen ska avsluta sin skottsträckning. Det behövs dock ytterligare 7-14 dagars långnattsbehandling i kombination med växlande utomhustemperaturer för att generna som styr vila och köldhärdighet ska aktiveras tillräckligt mycket för att få granplantorna att

uppnå en djup vila och bli lagringsbara tidigt på hösten. Den kortaste långnattsbehandlingen (7 dagar) gav en viss påverkan av gener som styr vila och frystolerans jämfört med obehandlade kontroller, men effekten var svag jämfört med de längre behandlingarna (21 och 28 dagar). Eftersom plantskolorna i normala fall långnattsbehandlar plantorna under 21-35 dagar och genuttrycken börjar bli tydliga först när behandlingen är avslutad så är analyser av genuttryck inte användbara för att avgöra när långnattsbehandlingen pågått tillräckligt länge för att starta upp plantornas invintringsprocesser. Däremot skulle genuttrycken kunna användas för att fastställa plantornas vilostatus, förutspå utvecklingen av frystolerans, knoppsättning, risk för en andra skottskjutning på hösten samt förutspå när plantorna skjuter skott på våren. Ett indirekt mått på när långnattsbehandlingen i plantskolorna kan avslutas skulle kunna vara att man med eventuellt tidstillägg utgår från tidpunkten för skottsträckningens avslutning.

På senare år har intresset för att plantera Douglasgran (*Pseudotsuga menziesii* (Mirb.) Franco) ökat i Sverige, men tillgången på lämpliga provenienser har varit begränsad. Douglasgranen är väldigt känslig för både höst- och vårfrost vilket gör tidpunkten för knoppbrytning på våren till en viktig parameter vid val av proveniens. För att undersöka skillnader i tidpunkt för knoppsprickning hos några provenienser av douglasgran gjordes ett fältförsök, samt en växthusstudie där även en lokal proveniens av gran ingick. Genom att knopp-sprickningen i fält- och växthusförsöket gav samstämmiga resultat skulle växthusförsök kunna förenkla proveniensvalet och vara ett komplement till utläggning av fältförsök. Tidigare försök har visat att inlandsproveniensen Three Valley har ett invintringsmönster som överensstämmer med en lokal mellansvensk gran (lat. 57°00’N). Detta i kombination med att Three Valley-proveniensen också uppvisade ett likartat knoppbrytningsmönster som den lokala granproveniensen i denna studie, öppnar möjligheter för ett lyckat resultat i fält i södra Sverige.

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