Final Report

The application of cDNA microarray technology for unravelling molecular events underlying dormancy and cold hardiness in forest tree seedlings. (COLDTREE)

A first step towards the development of molecular diagnostic tests for cost efficient reforestation and nursery logistics.

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1. Introduction

1.1 Description of the problem

Sustained yield from Europe's commercially exploited forests requires a supply of millions of seedlings annually. The planting stock for reforestation and urban horticulture, almost 1.7 billion tree seedlings and ornamental woody plants comprising a total value of about 2 billion Euro, is mainly produced by European forest tree nurseries. These nurseries rely on a tight scheduling of operations, to be able to deliver vital seedlings to the planting site. A critical step in a modern nursery production chain is the transfer of seedlings to cold or frozen storage. Cold storage is required to prevent winter damage, especially in containerised seedlings, to maintain planting stock in an inactive condition, and to ensure plant supply for geographically distinct planting sites, a requirement for large scaled or internationally operating nurseries.

Indoor storage has therefore become common practice, but poses a new dilemma for nurserymen. Efficient management requires that the handling of seedlings, such as transfer to cold storage, be carried out at the earliest possible time. However, lifting and storage of insufficiently hardened plants reduces vitality and may lead to cold damage, dehydration and fungal infection. To prevent this kind of damage, and its adverse economic effects on nurseries and end-users, it is of vital importance to be able to determine accurately the peak physiological condition for lifting or transfer.

Despite the economic importance of such decisions, nurserymen still predominantly rely on traditional (morphological) methods to identify this moment. Recently, several physiological measurement techniques have been proposed, and some of them have been used operationally. However, the number of nurseries in Europe utilising these techniques is limited, because the methods are either unreliable, labour intensive or technically demanding and the minimum test period (test dependent) can vary from 3 days to over 14 days. In nursery practice, where lifting opportunities can be severely limited by rainfall, frost and snow, such a delay may significantly reduce the number of plants lifted at peak physiological condition.

In addition to the onset of dormancy, dormancy release is also of economic importance. If plants growing out in the nurseries are put into cold storage too late in spring, they show evidence of damage, particularly to the root system. In spring, plants start reversing the processes that protect them during winter before there is a visible sign of regrowth.

So, efficient forestation and cost-effective nursery management require tools for rapid and reliable determination of the physiological condition of forest tree seedlings. To develop such tools, a thorough understanding of the cellular and molecular processes underlying cold hardiness is required. Unravelling the gene expression pattern as a seedling acquires the hardened state will reveal key processes that can be used as landmarks to describe the physiological condition of the tree. Eventually, this will result in molecular tests based on the presence or absence of specific messenger RNA's or proteins, that will allow a rapid evaluation of the physiological state and will facilitate forestation logistics. Such techniques are not yet available to forest tree nurseries and in this respect the forestry sector is lagging behind on horticulture and agriculture.

1.2 Physiological responses to cold stress

Plants living in the temperate zones display a strictly periodic growth pattern over the year. Absence of growth during autumn and winter is accompanied by frost tolerance in such plants.

Cold acclimation is a complex process comprised of several more-or-less discrete sub-processes, which are triggered by different environmental cues (Sakai and Larcher, 1987). Trees native to northern latitudes grow under conditions where marked annual temperature changes are observed and have developed physiological mechanism which result in strong periodicity in cold hardiness in phase with the climate (Bigras et al., 2001). Although cold acclimation and dormancy onset are influenced by temperature, for most native tree species at northern latitudes the primary environmental signal for induction of the cessation of shoot elongation and some degree of enhanced frost hardiness, is photoperiod i.e. the attainment of critical night lengths (Wareing, 1956; Thomas and Vince-Prue, 1997). The critical night length in plants is under strong genetic control and varies according to latitude and elevation

(Aitken and Hannerz, 2001). The attainment of tolerance to freezing temperatures occurs in response to near-zero thermal exposure, and to achieve maximal hardiness temperatures below freezing are usually required (Burr et al. 1989). The maximal capacity to cold acclimate then varies according to organ/tissue type, species, provenance, and exposure temperature. For example, aerial tissues of temperate conifers cold acclimate earlier and become more cold hardy than roots. The strategies that allow plants to survive freezing temperatures have been placed in two main categories, freezing tolerance and freezing avoidance (Levitt, 1980). Freezing tolerance in aerial plant tissues is facilitated by a mechanism where cellular water is lost to extra-cellular ice, and this mechanism is common in conifers. Tissues that avoid freezing stress do so by supercooling of intracellular water through concentration of the intracellular solution, this mechanism is common in deciduous species (Levitt, 1980). Dehydrins are a family of desiccation-induced 'antifreeze' proteins that are believed to play an important role in the development of such cold temperature tolerance (Close, 1997). Freezing of cellular water does occur and may result in cavitation (air filling) of the water-conducting conduits, this phenomena has been documented for deciduous trees but has been rarely reported in conifers, this is most likely due to differences in water-conducting vessel geometry (Sperry and Sullivan, 1992). However water-induced cavitation of conifers is common over winter, but its occurrence is due to increased tension within the plant, this tension occurs as evaporative loss is not fully replenished by soil water due to low temperatures impeding its uptake (Tyree and Sperry, 1989). The development of dormant and cold hardy winter states is also characterised by reduced photosynthetic performance, in conifers, causing suppression of both the capacity for and the efficiency of CO2 uptake (Leverenz and Öquist, 1987). This suppression increases inversely with photoperiod and is indicative of a reduction in the number of photosynthetic reaction centres and the de-epoxidation of violaxanthin that increases non-radiative dissipation of excess energy within the light-harvesting complex (Vogg et al. 1998). The organisational adjustments that allow maintenance of functional photosynthesis under low temperature conditions occur in the chloroplast thykaloid membrane, primarily through seasonal changes in the D1 protein complex (Vogg et al. 1998). In the root system the major environmental factor, influencing growth and hardening, is temperature. The effect of prolonged low (soil) temperature appears to be to alter plasma membrane permeability, most probably via membrane Ca2+ which plays an important role in stabilising membrane structure (DeHayes et al. 1997). Cell membrane damage (freeze-induced dehydration) causes enhanced ion (K+) leakage with recovery from 'non-lethal' injury through ion reabsorption via plasma membrane protein (ATPase) activity.

Thus, decreasing and continued low temperatures and changes in daylength cause cellular changes that result in observable physiological plant responses. Budset and growth cessation is primarily controlled by photoperiodic shifts, sensed by plants by phytochrome family light receptor molecules. The ability to withstand freezing temperatures is then achieved through both photoperiodic and thermal cues, which result in cellular membrane changes that allow plants to avoid or withstand cellular freezing damage and cope with light energy in excess of biochemical demand.

1.3 Molecular responses to cold stress

When plants are exposed to cold, a number of events at the molecular level take place. At lower temperatures, membrane fluidity decreases, causing membrane rigidification. This triggers a signal transduction cascade involving structural rearrangement of the cell followed by a transient cytoplasmic calcium increase, which in turn triggers gene expression (reviewed by Chinnusamy and Zhu, 2002; Chinnusamy et al.,2004). At lower temperatures, root water absorption may be reduced, which causes dehydration stress. Additionally, temperatures below zero can cause ice crystal formation in the apoplast, indirectly imposing osmotic stress on the plant cell. Molecular responses to cold and drought stresses have been shown to partly overlap, resulting in physiological adaptations that share similarities (Li et al., 2004; Seki et al., 2002).

Much work has been performed on the cold response of the model herbaceous plant Arabidopsis, using expression profiling of cold induced gene expression, or mutant screens to identify important regulator genes (reviewed by Shinozaki et al, 2003; Seki et al, 2003; Zhu, 2001). Many cold- and dehydration-responsive genes share a

conserved DNA sequence (CRT (C-repeat)/DRE (dehydration-responsive element)) in their promoter, which can confer both cold- and dehydration-responsive gene expression. A family of AP2-domain transcriptional activators, known as either the CBF (CRT binding factor) or DREB1 (DRE binding) proteins bind to the CRT/DRE element and activate transcription in response to cold or dehydration stress (reviewed by Thomashow, 2001; Shinozaki et al., 2003). Overexpression of the CBF/DREB1 proteins in Arabidopsis increases freezing and dehydration tolerance of the transgenic Arabidopsis plants, due to constitutive expression of a number of cold- and dehydration-responsive genes, and the accumulation of solutes that have cryoprotective properties (Jaglo-Ottosen et al., 1998; Gilmour et al., 2000).
Using gene expression studies with cold-shocked Arabidopsis plants, a number of cold induced genes have been identified. They can be grouped according to their expression pattern (for example, transiently induced or longer-term induced by cold, activated by CBF or CBF independently regulated), and belong to a variety of functional groups (Fowler and Thomashow, 2002). Prominent gene classes that were found to be long-term cold induced include transcription factors (including CBF and downstream targets), COR/LEA proteins (hydrophilic polypeptides thought to play roles in cryoprotection), and proteins with roles in sugar accumulation (Seki et al., 2002; Fowler and Thomashow, 2002).
In addition to the transient effect on gene expression caused by cold shock, exposure to non-freezing, low temperature is one of the environmental signals that trigger cold acclimation (hardening). The cold acclimation process allows plants to prepare themselves to cope with sub-zero temperatures, which might be lethal to non-acclimated plants. In woody plants, shortening photoperiod is one of the main signals for cold acclimation. Photoperiod interacts with temperature in controlling the level of hardiness reached during acclimation. In some cases dehydration has also been shown to induce freezing tolerance. Provenances are usually adapted to their local climate (sensitivity to photoperiod and temperature cues) in order to reach an appropriate level of frost hardiness in time for winter (reviewed by Li et al., 2004; Beck et al., 2004).
Hardening and dehardening are accompanied by a number of molecular and physiological changes. These include changes in lipid composition of the membranes, affecting membrane fluidity, synthesis of and accumulation of solutes and proteins that have anti-freeze properties, and an increased radical scavenging potential of the cells (Beck et al., 2004).

1.4 Implication of cold stress in practice

Overwintering seedlings is a crucial stage in nursery operations. In autumn after growth cessation the seedlings have to be stored in good condition for a period of up to 6 months before customers want the seedlings delivered. Also, seedlings that are to be grown for more than one season in the nursery need to be safely stored over winter. The winter climate may subject seedlings to severe stresses. There are several environmental conditions in winter which lead to severe drought stress.
Frozen ground reduces the water supply to the seedlings. This can become harmful if needles and other above-ground parts experience growth promoting temperatures and the stress is aggravated when it is windy. This kind of desiccation will most likely appear in late winter or early spring when foliage becomes active (Livingston, 1995)..
The main problem for all species in uncontrolled outdoor holding areas is the risk of being subjected to several freeze/thaw cycles and high cooling and thawing rates. These are both factors that are well known to increase winter injury in woody plants (see e.g. Sakai & Larcher, 1987; Livingston, 1995). A way to reduce the risk of production losses in winter is to store the seedlings in cold (+2 °C) or frozen (-3 to-5 °C) storage. Cold storage is therefore a key factor in the production of high quality seedling stock, which is important for the establishment success of new woodlands.
Tree seedling ‘stock quality’ assessments can therefore be defined in terms of their ability to predict successful establishment and survival, often after being subjected to prolonged and/or severe environmental stresses (Puttonen 1997). Predictive tests for cold tolerance are considered essential, as the determination of seedling physiological status by visual appearance or by calendar date is dependent on seasonal, ontogenetic or environmental variation (Fuchigami et al. 1982). Tests to determine cold hardiness

thus provide a basis for making decisions on lifting 'windows' and a reduction in losses after planting or cold storage (Burr et al. 1989; McKay 1992, 1997).
For plantation regeneration purposes a potentially important factor limiting yield and establishment success is the ability to withstand frost and in upland temperate regions it is most likely the limiting feature for provenance transfer (Cannell, 1984). In Sweden, one-year-old Scots pine seedlings from a southern seed orchard demonstrated delayed cold acclimation, decreased frost hardiness, an increase frequency of winter frost damage, and delayed autumn lignification when compared to natural stand progenies (Andersson 1994). Also, in many countries today increasing importance is being placed upon the use of and maintenance of local native material, often for biodiversity and cultural heritage reasons. However, we have few tools for the assessment of such population's ability to cope with a changing climate

Physiological assessments of cold hardiness have commonly been determined by assessing root membrane integrity through measurement of root electrolyte leakage (e.g. McKay and Howes 1992). Recent studies using techniques which do not require an artificial freeze step, such as bud dry matter concentration and impedance spectroscopy, have also been reported (Repo et al. 2000) though their relationship with field performance is currently uncertain. Despite these recent developments reliable and rapid methods for early screening are not available yet.

1.5 Gene activity as a molecular indicator for physiological status
In order to gain a better understanding of the components that play a role in triggering and accomplishing cold hardiness, and to aid in the development of reliable hardiness assays, a comprehensive study was performed to correlate physiogical and molecular responses in *Pinus sylvestris* and *Fagus sylvatica* seedlings. Despite the fact that beech and pine are commercially and environmentally important tree-species in Europe, little is know about the genetic background of their frost tolerance development.
During climate room and field trials at different locations, samples were simultaneously analysed for cold hardiness, dormancy, and gene expression programs, while photoperiod and ambient temperature was also recorded. This way, the relation among climate, physiological response and gene expression program could be quantitatively determined, and potential marker genes for cold hardiness could be identified.
Seedlings were grown in climate-controlled environments for the initial identification of relevant genes, followed by outdoor trials which were used to detect the effect of climatic conditions, geographical position and provenance on the underlying molecular processes.
The main objective in the COLDTREE project was to link the physiological features shoot cold tolerance and bud dormancy in *Pinus sylvestris* (L.) and *Fagus sylvatica* (L.) seedlings with vitality during and after cold and frozen storage and to correlate this with gene expression, by utilising powerful molecular techniques. Selected genes should then be developed as a 'first step' expert rapid, predictive molecular diagnostic test using PCR technology. Furthermore, the project contributed to our knowledge of the importance of different environmental cues in the development of cold tolerance and release from dormancy.
Furthermore, we report the first study across a north European ecocline, comparing both standard and local provenance material. Whilst physiological investigations of plant vitality have been reported, in many countries, for many aspects of the nursery supply chain, few comparisons at the provenance level have been made (Andersson 1985, 1994; Perks and McKay 1997; McKay and Morgan 1999. There is an urgent need to develop a deeper understanding of such species / provenance responses as future climate change creates additional challenges for forestry professionals (Bigras 2000). To this end a knowledge of the physiological response of 'local' and 'non-local' planting stock to different edaphic triggers (by dint of geography) can give us insight into the potential for adaptation of our existing forestry resources.
All data generated, both physiological, molecular, environmental and experimental, were combined in a relational database to allow data queries across the various parameters.

2. Materials & Methods

2.1 Plant materials

Pinus sylvestris

Scots pine of the seedlot A70 [SPA70], derived from a British seed orchard which is composed of tested clones with good progeny performance, was used as standard provenance in all pine experiments performed within this project. Cell grown SPA70 stock (1 y.o.) were grown in the UK at Bush Estate, Roslin, Midlothian (55° 51'N 03° 11'W, 170 m elevation) DIAS, Denmark (Scots pine: 55°18'N, 10°27'E) and Garpenberg, Sweden (60°17'"N Long: 16°12'E, 160 m elevation) alongside native provenance Lindås, Åmsele, and N401, for each country. Nursery cultural regimes were identical at all three sites, with plants grown under glasshouse conditions until the beginning of August, when they were removed to outdoor beds or to climate rooms. For provenance comparison, containerized Scots pine seedlings of three origins (A70, Sollerön (derived from 61°10'N, 408m elevation), and Åmsele (derived from 64°24'N, 19°19'E, elevation 250m)) were raised and nursed according to the standard protocol in 2001, 2002 and 2003 at Högskolan Dalarna Research station in Garpenberg, Sweden. In autumn 2003, 1-year-old Scots pine seedlings from three Swedish commercial nurseries were tested. (Table 2.1). The seedlings were sown in greenhouses in early spring 2003, moved outdoors and long-night treated in early summer.

Table 2.1. Geographical position for the commercial nurseries and origin of the provenances of the commercially grown Scots pine seedlings that were used for provenance comparison.

Nursery				**Provenance**			
Name	Lat.	Long.	Alt. (m)	Name	Lat.*	Alt.* (m)	No clones
Flåboda	56o34'N	15o11'E	130	Gotthardsberg	57o28'N	111	143
Nässja	60o16'N	16o48'E	55	Våge	65o48'N	440	52
Bogrundet	62o31'N	17o25'E	5	Bogrundet	65o54'N	440	44

* Average for the clones in the seed orchard

Fagus sylvatica

During summer seedlings were raised in accordance with normal nursery practice. The seed of test provenances were sown in week 19 (Year 1) and week 15 (Year 2) and cultivated in HIKO 265 trays in accordance with normal nursery practice.
For provenance comparison, seeds of three geographic provenances of common beech (*Fagus sylvatica L.*) were sown in Noordbroek, The Netherlands after stratification in three subsequent years, 2001, 2002 and 2003. The used provenances in 2001 and 2002 were the Dutch provenance NL2.1, the English provenance Greenhill and the Danish provenance Bregentved. The standard provenance Bregentved was obtained from the Danish Institute of Agricultural Sciences. In 2003 Greenhill was not available and was therefore replaced by a provenance from the same area. Cultivation was according to standard nursery practice. In autumn 2002 plants from seeds sown in 2001 were replanted for practical reasons and further cultivated according to standard nursery practice.
In 2003 beech seedlings from three commercial nurseries were sampled. These nurseries were all located in the South of the Netherlands, region Zundert. All three samples were the Dutch provenance 'Het Loo'.

2.2 Field and climate room experiments

Field experiments using pine

Seedlings were sampled at 2 week intervals from September to January (2001 – 2002, UK and Denmark) and for all three countries from September to January (2002 – 2003 and 2003-4). At each lift seedlings were removed to the laboratory for experiments to test seedling cold tolerance, with 15 replicates, with current years needles, assessed for each lift date x temperature combination. A minimum of 65 seedlings of each provenance was removed, from a randomised block design, for physiological tests aimed at evaluating dormancy depth (days to terminal budbreak: DBB) and hardiness

status (cold tolerance after artificial freeze testing (REL/SEL) of excised root and shoot parts (see below). After lifting all plants were stored at 4°C in sealed plastic bags, for a maximum of 5 days, until physiological and morphological tests were completed. Prior to testing plant systems (shoots and roots) were carefully washed in cold tap water then deionised water, to remove any remaining soil material. On arrival to the laboratory, the apical buds that were to be used for mRNA analysis were cut off from 50 seedlings. The buds were put into marked glass tubes, 25 buds per tube. The tubes were marked with a unique 8-digit identification number, sealed with a plastic cap and immersed into a container with liquid nitrogen pending RNA extraction.

Table 2.2. Geographical origin of the Scots pine provenances that were tested for frost tolerance.

Provenance name	Latitude	Longitude	Altitude, m	Harvested in
A70	56°32'N	03°32'W	160	seed orchard
Sollerön	61°10'N*		408*	seed orchard
Åmsele	64°24'N	19°19'E	250	natural stand

* average of 46 clones

Field experiments using beech

Beech stock of a standard seedlot [Bregentved] was grown in the UK, Denmark (year 1-3) and the Netherlands (year 2-3), alongside native provenance(s) for each country. In some years age effects were also studied

The bare-rooted beech seedlings (including different age material) were lifted in the nursery in week 37, 39, 41, 43, 45, 47, 49, 51 in 2003 and in week 2 and 4 in 2004. (in week 2 shoots only, because of frozen nursery soils). Except for the dormancy test in week 2, shoots of lifted seedlings were subjected to freezing and dormancy tests. Beech roots were not freeze-tested. In each lifting week 75 seedlings were cold stored at 4°C for field planting in April 2002 (UK) whilst in Denmark storage temperature lowered to –1°C in December. This allowed the two cultural practices of cool and cold storage to be assessed in order that the relationship between pre-storage physiological condition (frost tolerance, dormancy and gene expression) and storability (assessed as survival and growth) could be investigated.

Nursery cultural regimes were identical at all three sites, with plants grown under glasshouse conditions until the beginning of August, when they were removed to outdoor beds. Outdoor culture continued in containers in the UK and Netherlands whilst in Denmark seedlings were planted into cultivated beds to produce 'bareroot' seedlings, as this is the common practice in this country. At each lift seedlings were removed to the laboratory for experiments to test seedling cold tolerance, with 15 replicates, with current years needles, assessed for each lift date x temperature combination. A minimum of 65 seedlings of each provenance was removed, from a randomised block design, for morphological and physiological assessments. After lifting all plants were stored at 4°C in sealed plastic bags, for a maximum of 5 days, until physiological and morphological tests were completed. Prior to testing plant systems (shoots and roots) were carefully washed in cold tap water then deionised water, to carefully remove any remaining soil material.

On arrival to the laboratory, the apical buds that were to be used for mRNA analysis were cut off from 50 seedlings. The buds were put into marked glass tubes, 25 buds per tube. The tubes were marked with a unique 8-digit identification number, sealed with a plastic cap and immersed into a container with liquid nitrogen pending RNA extraction (by a common protocol) and further in-depth laboratory assessments of gene activity upon these extracted RNA samples.

At each site hourly estimates of ground and soil temperature were obtained. This data was collated for each site and the temperature expressed as accumulated chill sum (i.e. accumulated hour degrees <5°C). Accumulated chill sum and daylength have previously been shown to be primary drivers for the onset and development of cold tolerance and dormancy (Heide 1993a).

Climate room experiments using pine
Climate chamber experiments in the first trial season were designed to separate the processes of dormancy and frost hardiness development as far as possible, to allow for the selection of genes specific for the two phenomena. The experiments were performed with 1 year old *P. sylvestris* provenance A70. In the UK climate chambers photosynthetic photon flux density (PPFD) at the leaf level was 350 µmol.m^{2}.s^{-1}.
Three climate regimes have been be applied:
Fixed daylength: 16 h. Decreasing temp: 15 > 5 °C
Fixed temperature 15°C,. Decreasing daylength: 16 h > 6 h
Fixed daylength: 16 h. Fixed temperature: 15 °C.

The second trial season was devoted to investigation of the interaction between temperature and daylength. Four climate regimes have been applied:
Climate 1 (control): long day (17h), high temperature (17/15°C)
Climate 2: decreasing daylength (17h/8h), decreasing temperature (17/13°C > 5/5°C) (non-linear stagger).
Climate 3: 'SCOLD': removal from Climate 2 at week 40 to 6°C day/night and equivalent light regime, tested week 41 & 42.
Climate 4: 'SWARM': removal from Climate 2 at week 46 to Climate 1 regime, tested week 47.

Sampling dates were week numbers 34, 36, 40, 41,42, 43, 46,47, 48, 49 and 52.
Physiological tests aimed at evaluating dormancy depth (days to terminal budbreak: DBB) and hardiness status (cold tolerance after artificial freeze testing (REL/SEL) of excised root and shoot parts) were conducted (see below).
For all treatments, 65 seedlings were randomly selected at each test occasion. On arrival to the laboratory, the apical buds that were to be used for mRNA analysis were cut off from 50 seedlings. The buds were put into marked glass tubes, 25 buds per tube. The tubes were marked with a unique 8-digit identification number, sealed with a plastic cap and immersed into a container with liquid nitrogen pending RNA extraction (by a common protocol) and further in-depth laboratory assessments of gene activity upon these extracted RNA samples.

At each lift seedlings were removed to the laboratory for experiments to test seedling cold tolerance, with 15 replicates, with current years shoots, assessed for each lift date x treatment (environment) combination. After lifting all plants were stored at 4oC in sealed plastic bags, for a maximum of 5 days, until physiological and morphological tests were completed. Prior to testing plant systems (shoots and roots) were carefully washed in cold tap water then deionised water, to carefully remove any remaining soil material.

Climate room experiments using beech
In the first experiment, conducted in Autumn/Winter of 2001-2, approximately 3000 seedlings were transferred to three climate rooms in week No. 33, where they were subjected to one of three different climate regimes, described below, until week No. 2

First year trial:

Climate 1 (Control):	Fixed temperature: Tday=15°C, Tnight=13°C. Fixed daylength: 15 h.
Climate 2:	Fixed temperature: Tday=15°C, Tnight=13°C. Decreasing daylength: 14 h > 6h
Climate 3:	Decreasing temp: 15>0ºC Tnight 2°C<Tday. Fixed daylength: 15 h.

Second year trial:

Climate 1 (Control):	Fixed temperature: Tday=15°C, Tnight=13°C. Fixed daylength: 17h.
Climate 2 (DECLINING):	initially set-up as climate 1, but daylength reducing by 1h and temperature reducing by 2°C every two weeks, Tnight 2°C<Tday until DL=8.5 h and Tday /Tnight =1/1°C.

Climate 3 (SCOLD): as climate 2. Seedlings tested after being subjected to a short cold period (5°C lower) in week 39 and 40 and subsequently after a 2 week 'recovery' period (week 41 and 42) in climate 2.

Climate 4 (SWARM): as climate 2. Seedlings tested after being subjected to a short warm period (5°C higher) in week 45 and 46 and subsequently after a 2 week 'recovery' period (week 47 and 48) in climate 2.

Climate rooms operated with a photosynthetic photon flux density (PPFD) of approximately 600µmol m-2 s-1 at the 'canopy' level.
On each lifting week 75 additional seedlings were cold stored at 4°C. Storage temperature was then lowered to –1°C in December, a common practice in Denmark, and seedling trees maintained in storage at -1°C until removal for field planting, in April of that year. This allowed a study of the relationship between pre-storage physiological condition (frost tolerance, dormancy level and gene expression) and storability, assessed as survival and growth after planting.

Physiological tests were aimed at evaluating depth of bud dormancy, by assessment of days to terminal budbreak, and cold hardiness status, by assessment of electrolyte leakage of excised root and shoot parts, in response to artificial freeze tests:

A. Each sampling week, 2-cm shoot tip sections of 15 beech seedlings per climate were excised and frozen to –15°C and –25°C and 15 fine roots samples from the same seedlings were frozen to –5°C. 15 shoot and root control samples were held at +4°C. Shoot and root frost injury were evaluated using the conductivity method described in the common protocol, and denominated SEL and REL, respectively . Shoot frost tolerance was quantified as the difference in SEL(-25°C) artificial frost treated seedlings and control SEL(+4°C) seedlings, and denominated seldiff-25. Similarly, root frost tolerance was calculated as Reldiff-5.
B. Each sampling week 15 beech seedlings per climate were transferred to a greenhouse with favourable growth conditions (supplemented daylength = 18h, supplemented temperature = 20°C) for testing of bud dormancy status. The number of days to terminal bud break (DBB) of each seedling was recorded and dormancy status was calculated as the average DBB for each sampling week.
C. Each sampling week 50 terminal buds and 50 fine roots were collected and immediately frozen in liquid nitrogen for isolation of RNA.

The experimental design included 4 replicates, each sample consisting of material from 5 different plants. Thus, at each sampling date 20 different plants were analysed. In addition each sampling week 50 terminal buds and 50 fine roots were collected and immediately frozen in liquid nitrogen for isolation of RNA.
Regrowth was monitored in randomized trials consisting of 5 plots, of 10 plants each. Results were analysed statistically using analysis of variance calculations. In addition each sampling week 50 terminal buds and 50 fine roots were collected and immediately frozen in liquid nitrogen for isolation of RNA.

Frozen storage experiments using pine
Containerized Scots pine seedlings 1- and 2-year-old and provenances (Table 2.2) grown under natural conditions in Sweden were followed through the process of hardening in two successive years. At various time points during the hardening, different common physiological analyses were performed in order to get good physiological descriptions of the seedlings. The range of provenances ensured that seedlings in different stages of rest and of different levels of freezing tolerance were studied and described.

150 seedlings per provenance, age and occasion were randomly selected, packed into waxed cardboard boxes and put into storage (-5 °C) at three occasions in autumn 2003, in week 40, 44 and 48. The vitality of the stored seedlings was checked halfway through storage (week 7 2004) and at the end of storage (week 17, 2004). At each test occasion, fifteen seedlings per provenance and age were used in simple regrowth

tests. The seedlings were planted in 300 ml containers filled with peat and placed as 3 replicates of 5 seedlings in a greenhouse at a day length of 18 hours and 20/16 °C day/night temperature. Survival was registered and seedlings were considered dead if no root growth could be detected. 2-year-old A70 seedlings were not planted in these regrowth tests due to lack of seedlings. A more thorough root growth capacity test (Mattsson, 1986) was also made on 20 seedlings per provenance and age at the end of storage (week 16 2004).
Cell integrity of shoots and roots was assessed using the electrolyte leakage method. The degree of freezing tolerance was established with the freeze induced leakage method and described as SELdiff-25 for shoots and RELdiff-5 for roots. These figures, are in short the average leakage after freezing minus the average control (baseline) leakage, and they represent the damage that freezing to the actual temperature has caused the tissue. Freezing treatment of the shoots at -25 °C and control treatment in cold storage at 2 °C before assessment of the leakage was made individually on the apical 2 cm:s (without bud) of 15 seedlings per treatment. The roots were subjected to freezing at -5 °C and the same control treatment as the shoots was used. Roots were treated in five replicates with 3 seedlings per replicate i.e also 15 seedlings per temperature.

Provenance comparison for Pine
In 2002, 1- and 2-year-old and in 2003, 1-, 2- and 3-year old seedlings of the standard provenances were subjected to biweekly tests that started in week 36. The tests proceeded until winter conditions arrived in week 48. Seedlings were taken directly into lab from the outdoor holding area, a distance of approximately 10 – 20 m.

The seedlings from the commercial nurseries were tested three times per provenance at two weeks interval in autumn 2003. The seedlings from the nurseries were sent by post and arrived to Garpenberg on the Friday in the week before testing. At arrival to Garpenberg the seedlings were put into cold storage at 2 °C.

For all treatments (ages and provenances), 65 seedlings were randomly selected at each test occasion. On arrival to the laboratory, the apical buds that were to be used for mRNA analysis were cut off from 50 seedlings. The buds were put into marked glass tubes, 25 buds per tube. The tubes were marked with an 8-digit identification number (Appendix 1), sealed with a plastic cap and immersed into a container with liquid nitrogen. The following day the tubes were put into a box filled with dry ice and transported to Högskolan Dalarna in Borlänge, approximately 80 km north of Garpenberg, and placed in a freezer at -80 °C. Due to lack of seedlings in 2003, only 30 apical buds, instead of 50, were collected from the 2- and 3-year-old A70 seedlings. At the very last test occasion (week 48 in 2003) the apical buds from the 2- and 3-year-old seedlings that had their shoots frozen to -25°C were stored individually in order to be able to link the shoot freezing tolerance to the gene expression for each individual seedling.

On 30 of the seedlings without buds the root and shoot freezing tolerance was assessed using the freeze-induced electrolyte leakage method. The remaining fifteen seedlings with buds were used in bud break tests in order to establish the degree of dormancy.

Provenance comparison for Beech
Experiments 2002-2003
In 2002/2003 1- and 2-year-old seedlings of all provenances were used for testing. The bare-rooted seedlings of all provenances and the two ages were lifted 8 times during the season, in week 33, 35, 37, 40, 42, 45, 48 2002 and in week 6 2003. For each age/provenance combination 20 shoots were used. For mRNA analysis apical buds of 20 plants were sampled (3 buds/plant on average) and immediately stored in liquid nitrogen. The buds were stored at –80°C until mRNA analysis. For mRNA analysis week 33 is missing. Before determination of physiological parameters, like SEL and EC plants were packed in closed plastic bags and stored at 4°C until testing, that normally started the next day. Before further testing the plants were rinsed with tap water to

remove sand and dirt. The lifted seedlings were at random divided in two groups of 10. Length (cm), SELdiff-25°C (%) and EC (µS/cm) were measured.

Experiments 2003-2004
In 2003/2004 1- and 2- and 3-year-old seedlings of all provenances were used for testing. The bare-rooted seedlings of all provenances and three ages were lifted 7 times during the season: week 36, 39, 42, 45, 48, 51 (2003) and week 3 (2004). 50 seedlings of each provenance/age group were used. The apical buds were sampled and immediately frozen in liquid nitrogen. The buds were stored at –80°C until mRNA analysis.
The seedlings were lifted from two experimental plots, 25 seedlings/plot, and handled separately. Length (cm), SELdiff-25°C (%) and EC (µS/cm) were measured.

Commercial nursery samples 2003-2004
Seedings, Dutch provenance 'Het Loo', were obtained from three commercial nurseries in the south of The Netherlands, region Zundert. The seeds were sown in either 2001 ('Piet') or 2002 ('Marc' and 'Jos'). The seedlings were lifted in week 40 and 49 and treated as described above.

2.3 Physiological measurements and analysis

Cold Tolerance Assessments
The root (R) and shoot (S)ELdiff of seedlings was determined by subjecting samples to a specific freeze temperatures for roots at –5°C and for shoots at –15°C (UK and Denmark material) and –25°C (UK, Denmark and Swedish material) in a programmable freezing cabinet. After comparison against a baseline (no freeze) control value, obtained after a +4 °C treatment data was then analysed. Both baseline EL and frost hardiness was determined on the same set of plants for each temperature at each lifting date. For each test batch, standard samples of the root or shoot system were taken. Plants were rinsed in de-ionised water and baseline measurements were obtained by electrolyte leakage of excised plant parts, for unfrozen individuals, following the method of McKay (1992). The conductivity of the ions which had diffused from the test sample into the surrounding distilled water was measured after 24 h and again after autoclaving (10 min, 110 °C) which lysed the cells, once samples had been allowed to cool to room temperature. Conductivity was measured using a platinum electrode (k = 1.0) with built-in temperature compensation. The conductivity after 24 h was expressed as a percentage of the autoclaved value using the following equation:

[24 h value - conductivity of distilled water] x 100 = EL (%)
[Autoclave value - conductivity of distilled water]

Frost hardiness was assessed by placing rinsed root samples in glass bottles which were capped and subjected to a simulated frost temperature in a programmable freezing cabinet. Samples were cooled from 15 °C to 1oC in 1 h, cooled to a set minimum temperature at 2oC h-1, a rate which avoids rapid cooling injury by intracellular ice formation (Levitt, 1979), maintained at the set temperature for 3 h, warmed at 3 °C h-1 to 1oC and taken back to a maintained temperature of 15°C. The freeze temperatures were selected to provide cold tolerance estimates useful for assessment of the plants ability to withstand operational cold storage (-15 °C shoots and –5 °C roots) and to identify (the period of) maximum cold tolerance development in the shoot (–25 °C). Results were then calculated as above and expressed as the product of post-freeze values minus the baseline (control) estimate: the application, validity and interpretation of SELdiff has previously been published (Brønnum et al. 2004).

Bud break (dormancy) test
15 seedlings per treatment were replanted in 300 ml containers filled with peat. The seedlings were placed as 3 replicates of 5 seedlings in a greenhouse at a day length of 18 hours and 20/16 °C day/night temperature. The state of the apical buds was recorded three times per week. Time to bud break was assessed using a scale

(adapted from Murray et al (1989), applied to the apical bud of each plant. The buds were deemed to have broken when green needles or leaves had become visible (Table 2.3). The bud break tests were finished 90 days after planting when trees were considered as unable to burst (cf. Heide 1974, 1993a, b).

Table 2.3. Classification of apical bud and first whorl bud burst for seedlings in bud break tests – standard ColdTree protocol.

SCORE	BUD STATUS
1	Dormant bud in winter state
2	Bud swelling
3	Shoot elongating, green needle tips visible
4a	Shoot elongating, all needles visible, pairs separated
4b	Next years terminal bud formed and clearly visible

EC determination
EC was measured at room temperature using a conductivity meter. EC was measured at 5 cm above root collar in 0.5-year old plants and at 10 cm above root collar in 1.5- and 2.5-year old plants. Ten replicates were measured.

Statistical Analyses
The seedlings used in this study were replicated (by lift date) at each location using a randomised block design. Transformed values were used to homogenise variance before analyses. The effects of species, lift date and their interactions on EL estimates (transformed to arc-sine square root) were analysed using ANOVA (SAS 1989). Least Squares Mean (LSMEAN) was used to calculate REL averages for each treatment because of unequal observation numbers between sampling units. DBB data was analysed using ANOVA and the Kaplan-Meier method.
For beech field trials seedlings were replicated (by lift date) at each location using a randomised block design. Transformed values were used to homogenise variance before analyses. The effects of species, lift date and their interactions on EL estimates (transformed to arc-sine square root) were analysed using ANOVA (SAS 1989). Least Squares Mean (LSMEAN) was used to calculate REL averages for each treatment because of unequal observation numbers between sampling units. DBB data was analysed using ANOVA.

2.4. Gene expression profiling and analysis

2.4.1 Pinus sylvestris

RNA isolation and cDNA library construction
In order to prepare cDNA libraries, RNA was isolated from apical buds from *Pinus sylvestris* seedlings that were grown in the field in the Netherlands. RNA isolations were performed according to Chang et al., (1993). For library construction, mRNA was extracted using Dynabeads (Dynal Biotech, Germany). Cold acclimated apical buds harvested in December 1998 in the Netherlands were used to prepare a cDNA expression library (EL), using the λ-ZAP II system (Stratagene, The Netherlands) following manufacturers instructions.
In order to make subtraction libraries which were enriched for genes involved in cold tolerance, three additional mRNA samples were prepared. These were from buds harvested in February 2001, which were cold acclimated in the field (quiescent), buds harvested in April 2001, which had already resumed growth (released), or buds from seedlings that had been exposed to an additional short-term low temperature treatment in February 2001 (cold treated: artificial decreasing temperature regime from 20°C to -10°C in steps of 5°C), respectively. Two different cDNA subtraction libraries were made using the PCR Select cDNA Subtraction Kit (BD Biosciences Clontech, Europe) according to manufacturers' instructions. In order to enrich for quiescence related genes, RNA from the quiescent sample was used as tester whereas a mixture of equal

portions of RNA from the cold treated and the release samples was used as driver, producing the quiescent enriched library (QL). For enrichment of cold induced genes, RNA from the cold treated sample was used as tester whereas a mixture of equal portions of RNA from the quiescent and the release sample was used as driver resulting in cold enriched library (CL). The Advantage PCR Kit (BD Biosciences Clontech) was used to amplify of the libraries. PCR conditions were according to the manufacturer's protocol. The products were randomly cloned into the pGEM-T Easy Vector (Promega, The Netherlands).

Preparation of the pine cDNA microarray
Colony PCR with universal primers (M13 forward and reverse) was performed on individual bacterial colonies, in order to generate PCR products from individual cDNA inserts. Approximately 2000 cDNA fragments larger than 500 bp were selected for further analysis. These PCR products were purified using Multiscreen FB 96 wells plates according to the spin protocol provided by the supplier (Millipore, USA), and subjected to DNA sequence analysis using the big dye terminator cycle sequence ready reaction kit (Applied Biosystems, USA). The resulting sequence data were used to compare the sequences to GenBank in order to predict gene function. To determine the number of unique genes and to reduce the amount of redundancy on the microarray, a contig analysis was performed of the EST sequences (software; Seqman 11, DNASTAR). The 1900 EST sequences assembled into 1080 contigs, Some sequences were found very frequently in the different libraries, resulting in relatively large contigs. From the 1900 clones 1451 genes were selected for spotting on the microarray, attempting to include the maximum number of different genes, or different fragments from the same gene on the microarray. These included 803 clones from the EL, 351 from the CL and 297 from the QL cDNA library. Additional genes were added to a total of 1536 genes. These included fragments (amplified fragment length polymorphisms: AFLP fragments) of genes that were differentially present in a PCR assay performed during a previous EU project (FAIR CT95 0497) and some genes that were described in the plant literature to be associated with stress or dormancy. Additionally a number of control genes were spotted on the array. In order to increase the reliability of the produced data, all clones were spotted onto the microarray in duplo, on different regions of the microarray, as described by Soeda et al. (2005).

Hybridisation of the pine microarray
To be able to compare all microarray results a common reference was used in each hybridisation. The common reference was made from a mixture of pine buds that were harvested at different times in winter and early spring in Denmark and the Netherlands, to avoid a biased representation of certain RNA species in the common reference. For labelling the tester (RNA from samples from the different experiments) 40µg of total RNA was used. For the common reference 80µg total RNA was used per hybridisation. Fluorescent labelling, pre-hybridization and hybridization were performed according to Soeda et al. (2005). Gene Frames used for this array were 1.5 x1.6 cm (Abgene, Epsom, UK). Slides were scanned on either a Scanarray 3000 (Perkin Elmer), or a Scanarray express HT (Perkin Elmer), and raw data were exported to MS excelTM for analysis.

Data collection, normalisation and statistical analysis
Non-hybridizing yeast clones were used to calculate background levels for each hybridisation. After subtraction of background, data were corrected for relative dye intensity using median normalisation. The resulting hybridisation intensities and ratios were exported to excel for further analysis. To ensure that only reproducible differences in gene expression were included in the analysis, spots with hybridisation intensity below 1.5 times background were excluded from the analysis, and average values of duplicate spots were used.
All expression data obtained for pine using 50 different samples and 1500 different genes were combined in a single MS excelITM sheet together with the relevant physiological data. Prior to analysis, the physiological data were transformed to make them more compatible with the gene expression data. The % electrolyte leakage (in SEL diff-25°C and REL diff -5°C) was subtracted from 100 to obtain % electrolyte

retention (SER diff -25°C, RER diff -5°C), and subsequently divided by 10. The cold index (number of hours where ambient temperature was below 4 degrees C) was divided by 300, the cold index previous fortnight (number of hours during the previous two weeks where ambient temperature was below 4 degrees C) was divided by 100, and day length was divided by -4. This way, the values of physiological data were in the same range as the gene expression ratios, and increased over time. This allowed clustering of physiological data with genes that similarly increased in expression over time, facilitating marker identification. After this, all data was imported simultaneously into GeneMathsTM and analyzed together using statistical tools such as hierarchical clustering and principal component analysis. Principal component analysis (PCA) is a singular value decomposition (SVD) based method with the objective to determine a new coordinate system. This data transformation results in two or three components explaining the majority of the variance in the data (for example age of the seedling, location or provenance). A biplot represents both the row and column reduced information. This strategy enables a simple representation and facilitates interpretation of complex multidimensional data (Chapman et al., 2001).
Chip to chip variation among hybridisations was monitored using repeated experiments and swapped dye experiments. Furthermore, all cDNAs were spotted in duplicate on each array, while the presence of multiple cDNAs representing the same gene allowed monitoring of the internal chip variation.

2.4.2 Fagus sylvatica

Plant material for cDNA library construction.
Apical buds were collected from two-year-old seedlings of three different provenances grown in the field in The Netherlands (Zuidbroek). The beech provenance Bregentved was used next to provenance Greenhill and NL2.1. Forty seedlings were lifted every two weeks from September (week 35, 2002) until February (week 06, 2003). Buds for RNA isolation were frozen in liquid nitrogen and part of the sample was used to assess frost tolerance. Apical buds from all three provenances were mixed together and samples from September (week 37, 2002), where the plants were proven to be frost sensitive, and from December (week 48, 2002), where the plants have reached maximal frost tolerance, were used for construction of subtraction libraries. The remainder of the samples was mixed together and extracted RNA was used as a reference probe for microarray hybridisation.

Preparation of the beech cDNA microarray
Total RNA was isolated from the September and the December apical buds according to Chang et al. (1993). mRNA was isolated using the Oligotex mRNA Kit (Qiagen, The Netherlands). In order to enrich for cDNAs present in either one of the samples, subtractions in both directions were performed using the PCR Select cDNA Subtraction Kit (BD Biosciences Clontech) according to the manufacturer protocol. The Advantage PCR Kit (BD Biosciences Clontech) was used for amplification of the libraries. Amplified products were cloned in the pGEM-T Easy Vector (Promega, The Netherlands).
Greenomics (Wageningen, The Netherlands) performed DNA sequencing of randomly selected clones from both libraries. DNA sequence identity was established using BlastX. DNASIS Ver. 2.6. (Hitachi Software Engineering Co.) was used for contig analysis. A selection of 343 different PCR fragments was spotted in duplicate on Nexterion Slide E (Scott Nexterion) according to the protocol of the manufacturer. In addition, PCR fragments functioning as negative controls and the complete coding sequence of the firefly luciferase gene, used as normalisation feature, were spotted multiple times.

Hybridisation of the beech microarray
For hybridisations samples were used derived from field and climate room trials (described above) from the following series:
Field trial series 406 one-year-old Bregentved, Denmark 2001
Field trial series 704 one-year-old Bregentved, UK 2001
Field trial series 418 two-year-old Bregentved, Denmark 2002

Field trial series 417 one-year-old Bregentved, Denmark 2002
Climate room trial series 409 one-year-old Bregentved, Denmark 2002
Climate room trial series 410 one-year-old Bregentved, Denmark 2002
Total RNA was isolated according to Chang et al. (1993) and purified with RNeasy (Qiagen, The Netherlands). Pure RNA, up till 20 microgram, complemented with 1 nanogram luciferase polyA mRNA was used for each individual labeling. Reference RNA was labeled with Cy3 and sample RNA with Cy5 using the CyScribe First-Strand cDNA Labeling Kit (Amersham Biosciences). After checking the integrity of the labeled cDNA using agarose electrophoresis, sample and reference cDNA were mixed and used for hybidisation of the microarray following the protocol supplied by the manufacturer of the slides. Cover slides and hybridisation chambers from Agilent Technologies (Palo Alto) were used. Hybridisation was allowed to continue overnight in an incubator where the slides were continuously rotating (Sheldon Manufacturing). Post hybridisation washes were according to the Nexterion protocol.

Data collection, normalisation and statistical analysis
Slides were scanned using a GenePix 4000B (Axon Instruments) scanner and total pixel intensities were assigned to the spots using GenePixPro software. Values were normalised by adjusting the Cy5/Cy3 ratio of medians of the luciferase signals to 1. Signals not reaching 3 times local background were filtered out. Finally duplicate expression ratios (2 log ratio Cy5/Cy3) were averaged and used for cluster analysis on the Spotfire DecisionSite for Functional Genomics (Spotfire Inc. Somerville USA).
In order to cluster frost tolerance data (SEL-15 values) together with the genes, data were normalised in the following way. Values were decreased by the average of that particular data series and subsequently divided by their standard deviation.

2.5 Validation and test development
RT-PCR
Total RNA was isolated according to Chang et al. (1993). Preparations were DNAseI (AP Biotech) treated and purified using RNeasy (Qiagen, The Netherlands). Half a microgram of pure total RNA was used for cDNA synthesis using Anchored Oligo(dT)23 (SIGMA, The Netherlands) and M-MLV Reverse Transcriptase (Invitrogen, Life Technologies). Dilutions of this cDNA were used for Realtime PCR using the qPCR Mastermix Plus for SYBR GreenI (Eurogentec, Belgium). Product formation was measured using the iCycler system (BIORAD Laboratories, The Netherlands). Primer sets are described in Figure 3.60. The signal obtained from the same batch of cDNA using primers homologous to Arabidopsis thaliana 18S rRNA was taken as a reference for normalisation. Relative changes in expression were calculated using the Gene Expression Macro (Version 1.1) supplied by BIORAD.

NALFIA based mRNA quantification
CTAB (Chang et.al. 1993) isolated total RNA was made DNA free using DNA-free from (Ambion (Europe) Ltd, United Kingdom) following the protocol of the manufacturer. Five hundred nanogram of total RNA was subsequently used for cDNA synthesis using M-MLV Reverse Transcriptase (Invitrogen). Anchored oligodT (SIGMA) has been used for priming the reaction. Reaction conditions were according to manufacturers conditions. Five percent of the reaction volume was used for amplification of two specific dehydrin sequences and a ribosomal sequence (18S) simultaneously using the Multiplex PCR mix (Qiagen). Characteristics of the genes and labels, attached to each of the gene-specific primers, are presented in Table 2.4. Labelled primers were synthesized by Biolegio B.V. (The Netherlands). The amplification protocol was according to the description of the manufacturer and the annealing temperature was set at 60 ºC. Twenty-five amplification cycles were performed using a standard PCR machine. 10 % (vol.) of the reaction mixture was applied to the NALFIA (van Amerongen and Koets, 2005) in a 100 microliter volume of running buffer (0.1M Borate buffer, pH8.8, 1% BSA IgG-free, 0.05% (v/v) Tween20, 0.02% (w/v) NaN3). All amplified fragments carried two different labels; one specific label for immobilization of the fragment at a specific location on the membrane, one common label (biotin) for attachment of carbonated streptavidin giving the visible signal (black line).

Table 2.4. Characteristics of the genes and labels, attached to each of the gene-specific primers in the NALFIA assay

Gene name	Expression characteristics	Labels used for immunodetection
Dehydrin 2 (Psdhn2)	Low expressionlevel at maximal frost tolerance levels	biotin – DIG
Dehydrin 5 (Psdhn5)	High expressionlevel at maximal frost tolerance level	biotin – FITC
18S (Controle)	Constant expression level	biotin – TXR

2.6 Construction of a relational database

Data from each partner was supplied as a Microsoft Excel spreadsheet with a number of worksheets containing the data/meta-data.
The partners had agreed templates for each dataset, so the spreadsheets were relatively consistent across the project for each type of data collected.
By studying the data and applying the standard relational normalisation process it was possible to create a database structure that represented the Cold Tree data with logical links and no repeated data. After uploading data manual checks had to be made against the database to ensure all data had been uploaded correctly.

Relational databases were chosen as other forms of database (Object Orientated, XML) are either expensive or offer poorer performance characteristics. Also relational databases are commonplace, others require specialised skills and are therefore more expensive to maintain. For Cold Tree the data was loaded into a Microsoft Access database, which features a built in drag and drop query builder. This means that even novice Access users can develop queries. This tool also allowed Rapid Application Development (RAD) – there was in built support for building Graphical User Interfaces (GUI) with minimum amounts of computer code.

3. Results

3.1 Field trials using pine

Cold Hardiness Assessments

Over the three years of study material from the Danish study site showed consistently lower SEL values primarily due to higher baseline estimates (Figure 3.1, 3.2a, 3.2b). Despite the resultant lower early-season values obtained for SELdiff the interpretation of the physiological data is not impaired. This is because of primary importance is the seasonal development of cold hardy tissues and the date at which a target threshold level of leakage is observed (e.g. ≤10%). The intercomparison of sites based on threshold analysis is therefore still valid, and the analysis and comparison with molecular data, which is based on the timing of early onset, maximum decrease and full cold hardiness development is still appropriate as these trends are discernible.

In the first year of study (Year 1) for all provenances a significant effect of lifting date was evident (Figure 3.3). A significant difference was evident between Danish and UK A70 seedlings in the development of shoot cold tolerance, through assessment of SEL after artificial freeze testing at -25oC, ($P<0.01$, Figure 3.3a, d). For Danish grown A70 material cold tolerance to -25oC developed by week 49 (SEL below threshold value of 5%) whereas UK material did not become tolerant to this artificial freeze temperature throughout the duration of the experiment). For both Danish and UK grown A70 seedlings cold tolerance to -15°C developed by week 45. The 'native' Danish Lindås stock was cold tolerant to -15oC at week 45, mirroring the cold hardiness of A70 grown at this location, and tolerance to -25oC was reached at week 47. UK N401 material reached tolerance to -15oC at week 49 but did not fully develop tolerance to the -25oC artificial freeze temperature (Figure 3.3e). Within country comparisons between standard material (A70) and local material (Lindås or N401) revealed no significant shoot cold tolerance differences between provenances, irrespective of artificial freeze temperature.

In the second year of study similar trends were evident when comparing Danish and UK grown material. A significant effect of lifting date was evident for all provenances in all countries (Figure 3.4). Significant differences were evident between Danish and UK A70 seedlings in the development of shoot cold tolerance after artificial freeze testing at -25oC (Figure 3.2a, d; $P<0.01$). Again the -25oC tolerance threshold was not reached for UK grown material and Danish material reached this level of cold hardiness in week 51. For both Danish and UK grown A70 material cold tolerance to -15°C developed by week 43. The Danish Lindås stock was cold tolerant to -15oC at week 43, mirroring the cold hardiness of A70 grown at this location, and tolerance to -25oC was also reached at week 51. UK N401 material in the second year of study reached tolerance to -15oC at week 47 but did not develop tolerance to -25oC (Figure 3.4E). Within country comparisons between standard material (A70) and local material (Lindås or N401) revealed no significant shoot cold tolerance differences between provenances, irrespective of artificial freeze temperature. In the second year of study provenance differences were evident within UK material, and significant differences existed between the native UK provenance (N401) and the native Danish provenance (Lindås) in the rate of development of cold tolerance in the shoot.

For Swedish grown material differences were evident between the standard provenance and the native provenances, but native provenances did not differ in the rate or level of cold hardiness development in shoots (assessed at –25oC). In Sweden it was apparent that deep levels of frost tolerance are rapidly achieved: at Week 42 the shoot tolerance threshold was reached in response to –25oC artificial freeze tests.

In the third year of study similar trends were observed in terms of location and provenance effects on the development of root and shoot cold tolerance (Figure 3.5). Hardiness development across all years was in the order Sweden, Denmark, UK for cold tolerance in A70, with the exception of Year 3 where many deaths in Sweden compromised the quality of the data. Also in the third year age effects were investigated (Figure 3.6). At each site hourly estimates of ground and soil temperature were obtained. This data was collated for each site and the temperature expressed as accumulated chill sum (i.e. accumulated hour degrees <5°C) (Figure 3.7). Accumulated chill sum and daylength have previously been shown to be primary drivers for the onset and development of cold tolerance and dormancy (Smit-Spinks et

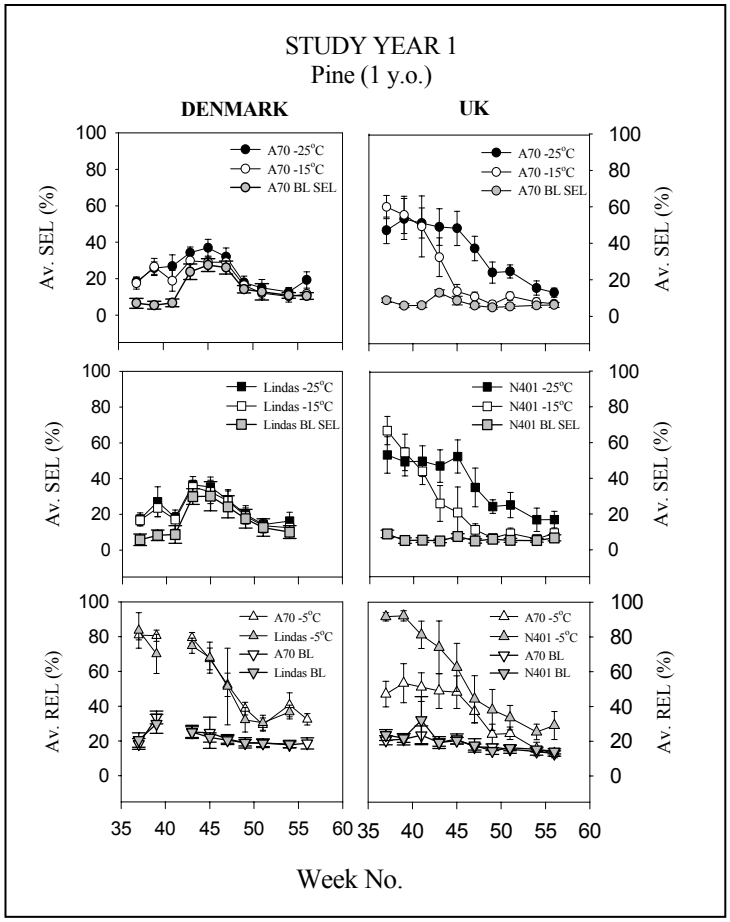

Figure 3.1. Seasonal physiological trends (observed in Year 1) in the average cold tolerance response of the shoots, as assessed by electrolyte leakage after artificial freezing (*n* = 15), of standard and local Scots pine provenance in Denmark and the UK relative to week of lifting.
Upper panels – baseline and average post-freeze shoot electrolyte leakage (SEL) of standard A70 provenance, middle panels - baseline and average post-freeze shoot electrolyte leakage (SEL) of local provenance material, bottom panels - baseline and average post-freeze root electrolyte leakage (REL).

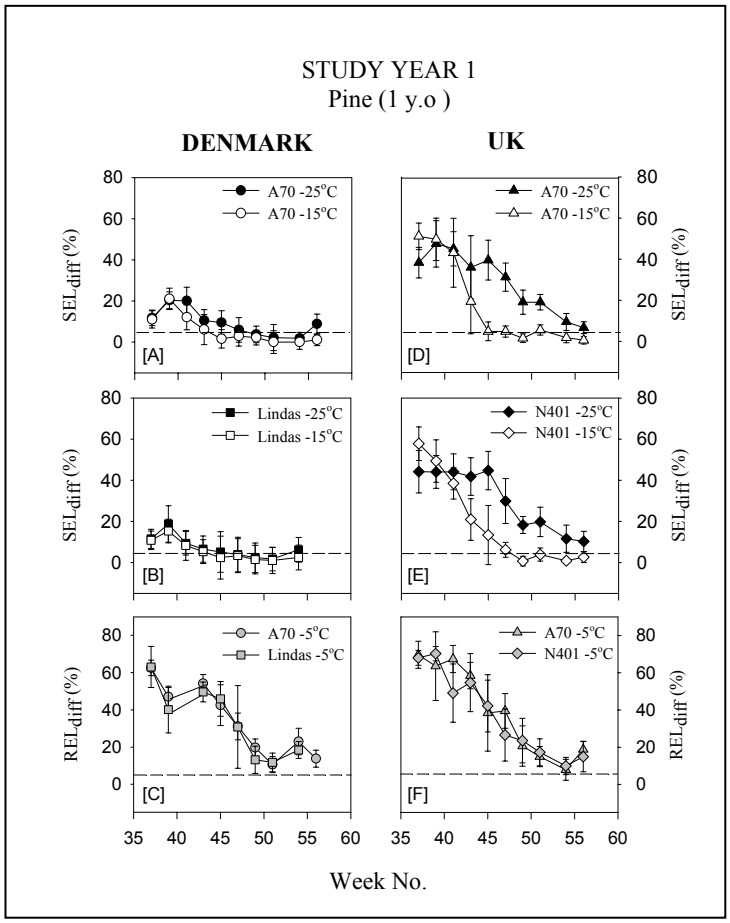

Figure 3.3. Seasonal physiological trends (observed in Year 1) in the average cold tolerance response of shoots (A-B and D-E) and roots (G-H), assessed by electrolyte leakage after artificial freezing (*n* = 15), of standard and local Scots pine provenance in Denmark and UK, relative to week of lifting.

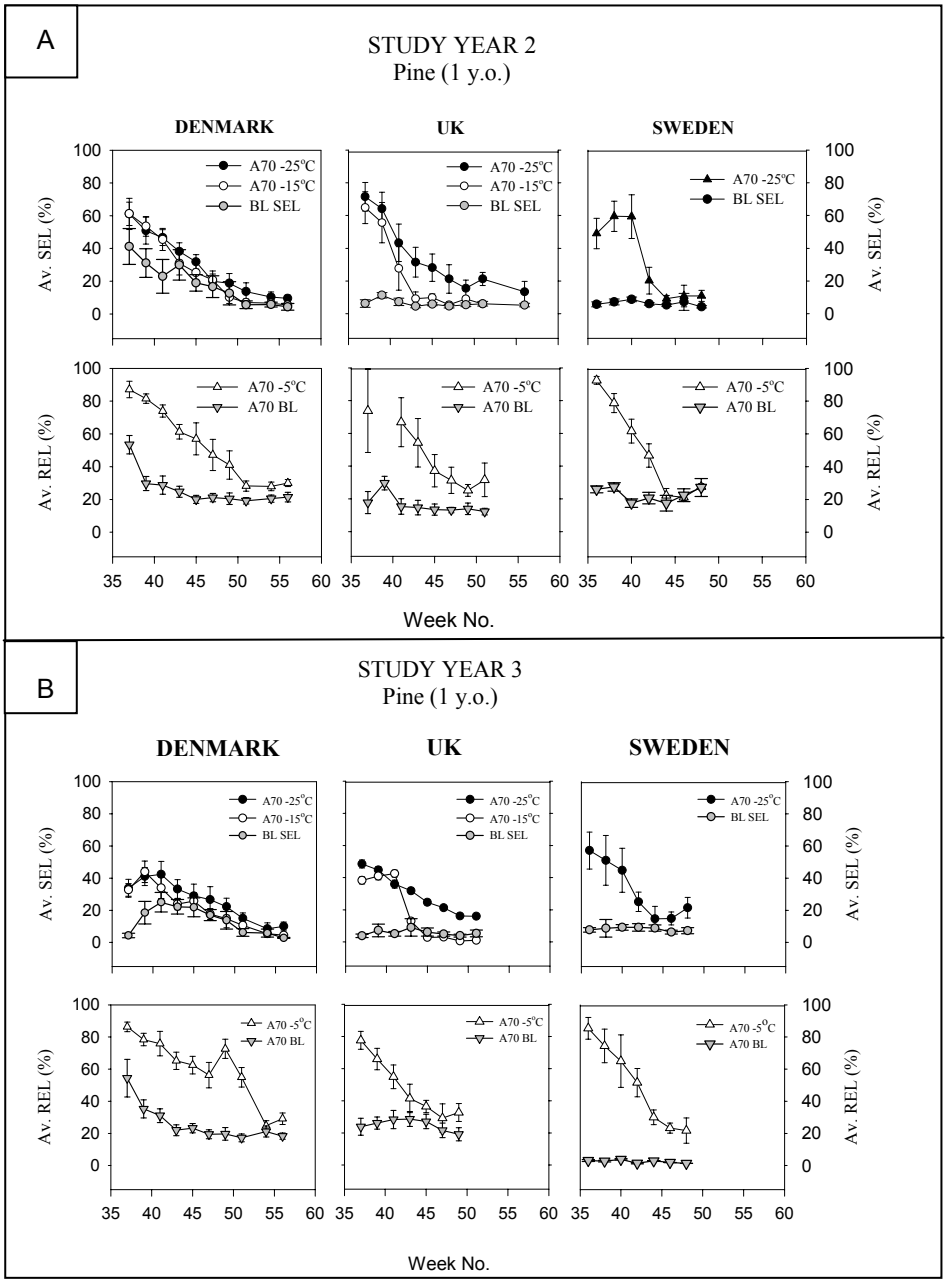

Figure 3.2
A. Seasonal physiological trends (observed in Year 2) in the average cold tolerance response of the shoot (top panels, standard provenance A70) and roots (lower panels, standard provenance A70), as assessed by electrolyte leakage after artificial freezing (*n* = 15). Baseline values are included.
B. Seasonal physiological trends (observed in Year 3) in the average cold tolerance response of shoots (top panels, standard provenance A70) and roots (lower panels, standard provenance A70), as assessed by electrolyte leakage after artificial freezing (*n* = 15). Baseline values are included.

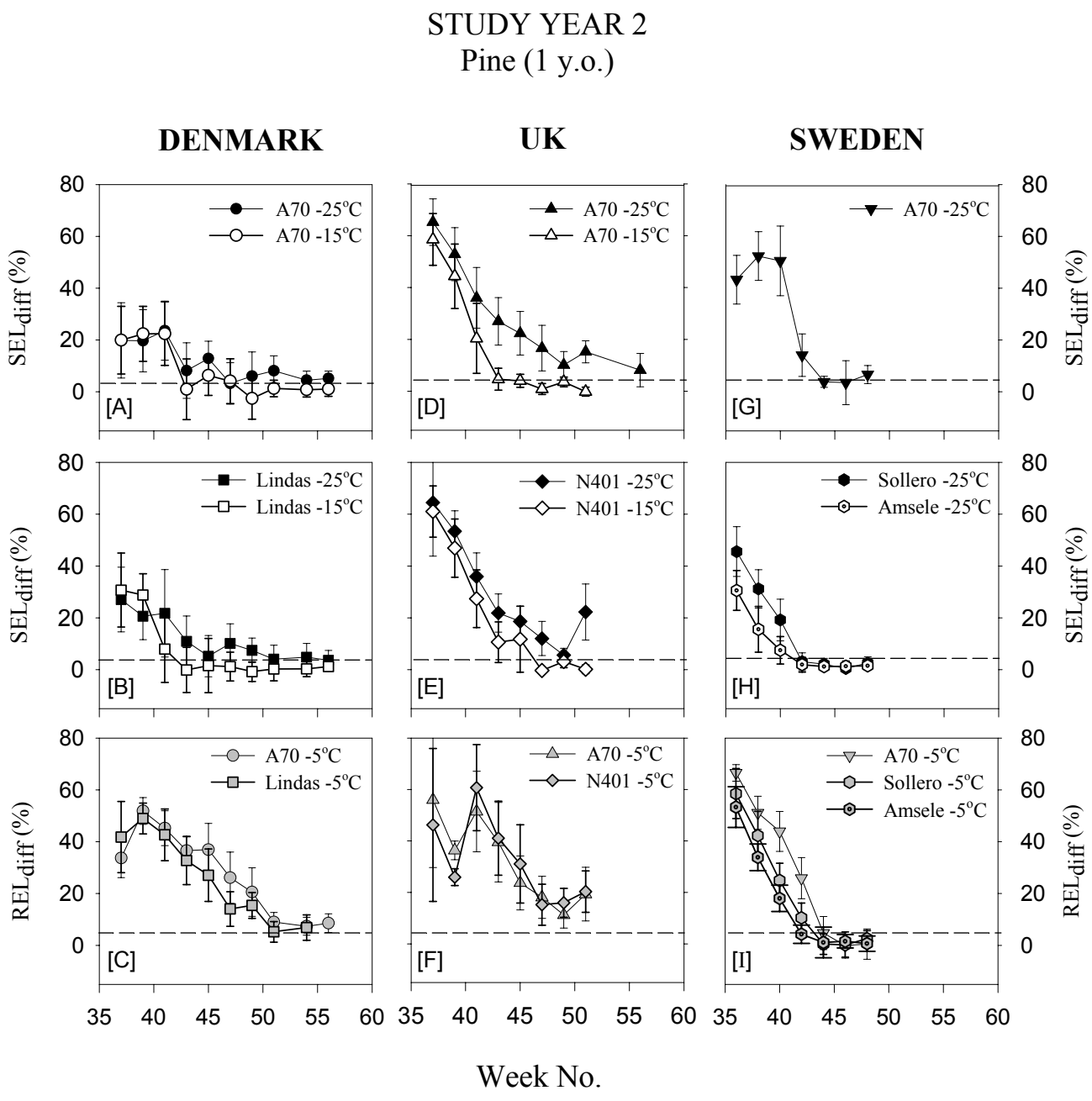

Figure 3.4. Seasonal physiological trends (observed in Year 2) in the average cold tolerance response of shoots (A-B, D-E and G-H) and roots (C, F, I), assessed by electrolyte leakage after artificial freezing (*n* = 15), of standard and local Scots pine provenance in Denmark, UK and Sweden, relative to week of lifting.

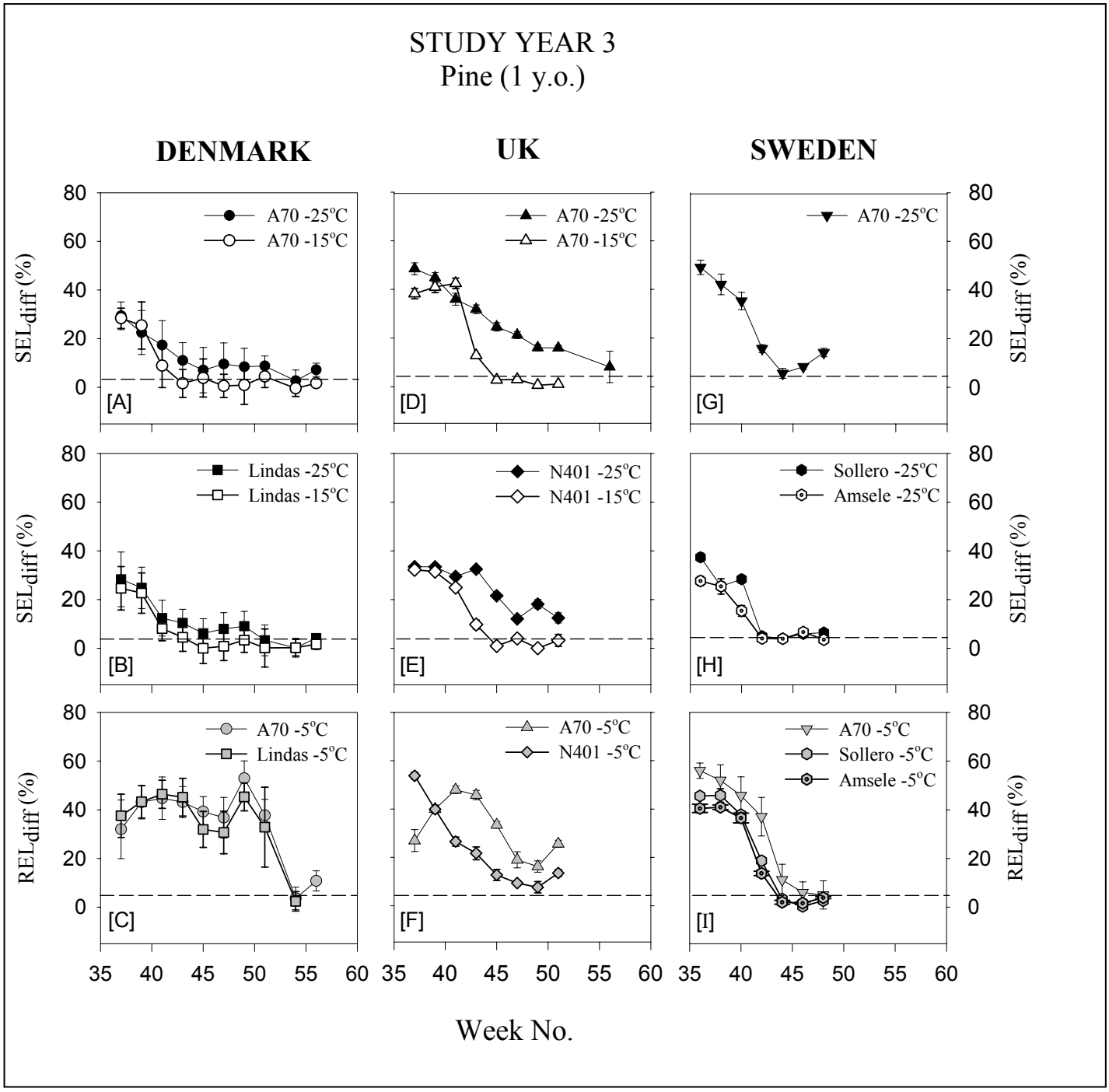

Figure 3.5. Seasonal physiological trends (observed in Year 3) in the average cold tolerance response of shoots (A-B, D-E and G-H) and roots (C, F, I), assessed by electrolyte leakage after artificial freezing (*n* = 15), of standard and local Scots pine provenance in Denmark, UK and Sweden, relative to week of lifting

al. 1985). In response to the development of cold tolerance noted in the declining temperature and daylength climate room environment, in Year 2 in-depth analysis of field environmental data based on the assumption that day/night thermal difference is the primary thermo-period cue led to a comparison for the UK (Figure 3.8).

Root Assessments
In study year 1 a significant provenance x environment effect was evident between Danish and UK grown A70 seedlings in the development of root cold tolerance, as evidenced through assessment of REL after artificial freeze testing at -5oC ($P<0.05$) (Figure 3.3 c, f). For Danish grown A70 material maximal root cold tolerance developed by week 51, whilst in the UK a similar level of root cold tolerance did not occurred until week 54. Within country comparisons between standard material (A70) and local material (Lindås or N401) revealed no significant root cold tolerance differences between provenances, on any sample date.
In Year 2 similar patterns emerged, with no significant differences between Danish and UK grown material. In the Swedish grown material the threshold tolerance of roots was achieved significantly earlier (week 42-44, Figure 3.4l).
In the third year of experiments there was no root tolerance developed in 2003, for UK or Denmark, in contrast to Swedish grown material (Figure 3.5).

Dormancy (DBB) Index
Low levels of dormancy (i.e. non-dormant) were noted in the first year of trial (Figure 3.9). In the following two years in the UK high dormancy was noted in the early weeks which then declined. Over the first two years no provenance effects were evident, in contrast to year 3 when A70 seedlings lost dormancy earlier than native N401 seedlings. As daylength is static across years variations in dormancy status can only reflect thermo periodic effects or, possibly, differences in ontogenetic stages between years. The best explanatory variables for these differences appear to be the accumulated sum of 5oC day night difference (Figure 3.8).

Cold Index
A significant difference was observed between Denmark, UK and Swedish climates, as evidenced by cold index ($P<0.01$). Regressions between cold index and physiological (shoot) variables were significant ($P<0.05$). A significant difference was observed between Denmark and UK climates (Year 1), as evidenced by cold index (Figure 3.7A, $P<0.01$). Daylength differences existed between the Swedish nursery site and Denmark/UK locations (Figure 3.7B). Seasonal effects were evident comparing between years of study within the UK, Denmark and Sweden (Figure 3.7A) and between sites. Thermal regime across year was such that Year 2 (UK, Denmark and Sweden) was the coldest, Year 1 was the mildest experienced in the UK and Denmark Year 3 was colder in the early phase than Year 1, though final thermal ‘load’ was similar (Figure 3.7A). As reductions in temperature and photoperiod are important both for cold hardening (Aronsson 1975) and the cessation of shoot elongation in Scots pine (Oleskyn et al 1998) it is possible that the initiation of cold hardening follows similar cues (Repo et al. 2001). Data from climate room trials (Year 2; see also) suggested that the day/night thermal difference (of 5oC) is the primary thermo period cue. This has led to in depth comparisons for the UK (to date) (Figure 3.6). Data presented shows evidence that correlation of physiological variables (by week number) are most closely approximated when plotted against daylength and that the cold index day/night 5oC thermal difference, with the exception of Year 2. Further analysis of data from all countries and with variation of thermal difference signatures is now required for complete understanding of the interactions of these cues visa-vie the development of cold tolerance in shoots. Seasonal physiological trends, with respect to year of experiment are summarized in Figure 3.10.

Outplanting survival
The correlation of SELdiff with outplanting survival is the ‘litmus test’ of the applicability of this measure as a logistical tool for use in the nursery supply chain, as it ensures that

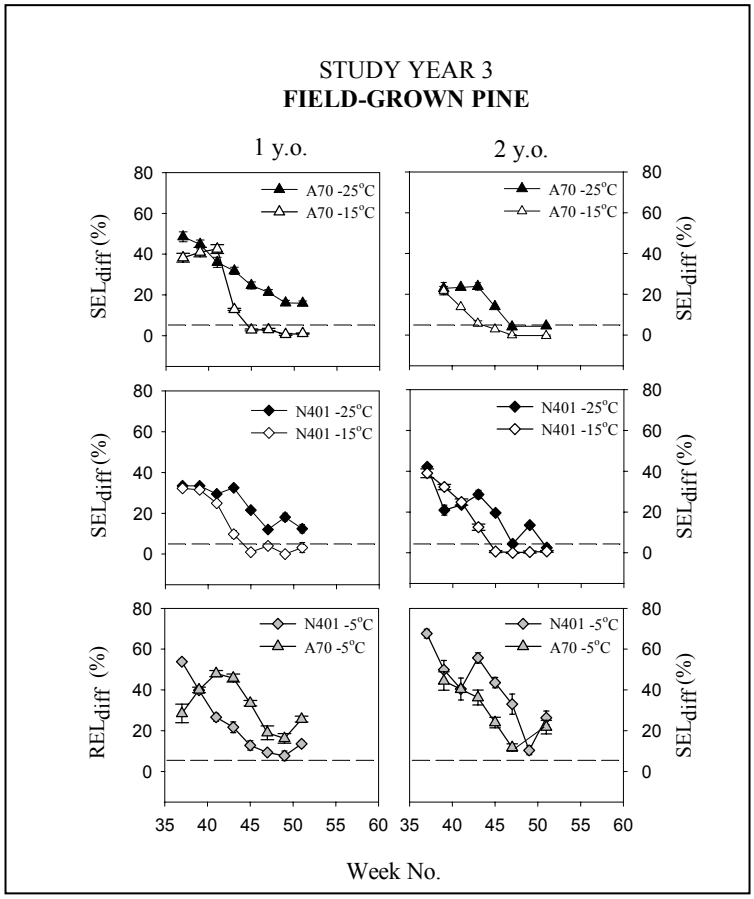

Figure 3.6 Seasonal physiological trends (observed in Year 3) in the average cold tolerance response of shoots (top and middle panels) and roots (lower panel) and the effect of seedling age, as assessed by electrolyte leakage after artificial freezing (*n* = 15), of standard and local Scots pine provenance in the UK, relative to week of lifting.

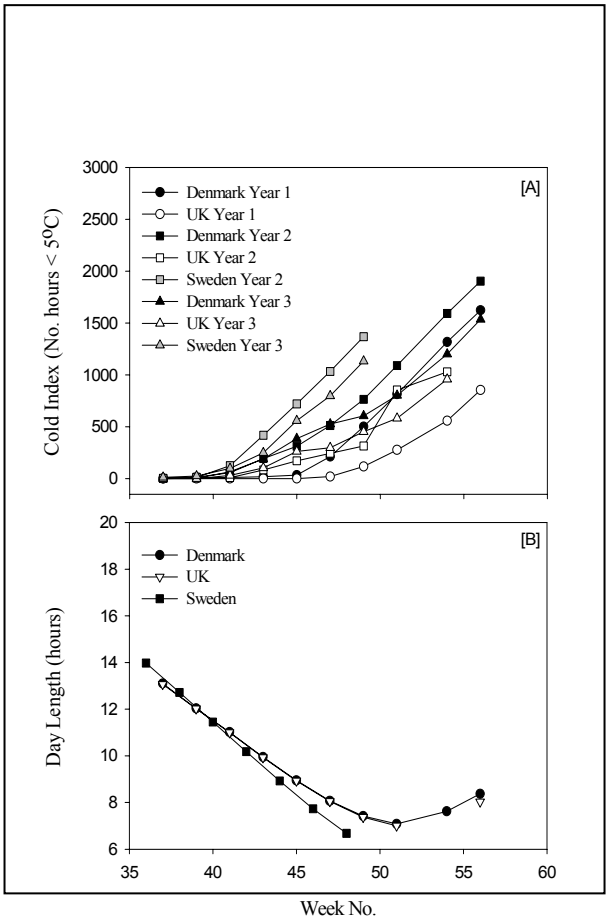

Figure 3.7. Seasonal trends, observed over 3 years of study, in a) cold index (calculated as accumulated hours <5°C) and b) daylength, in Denmark, UK and Sweden.

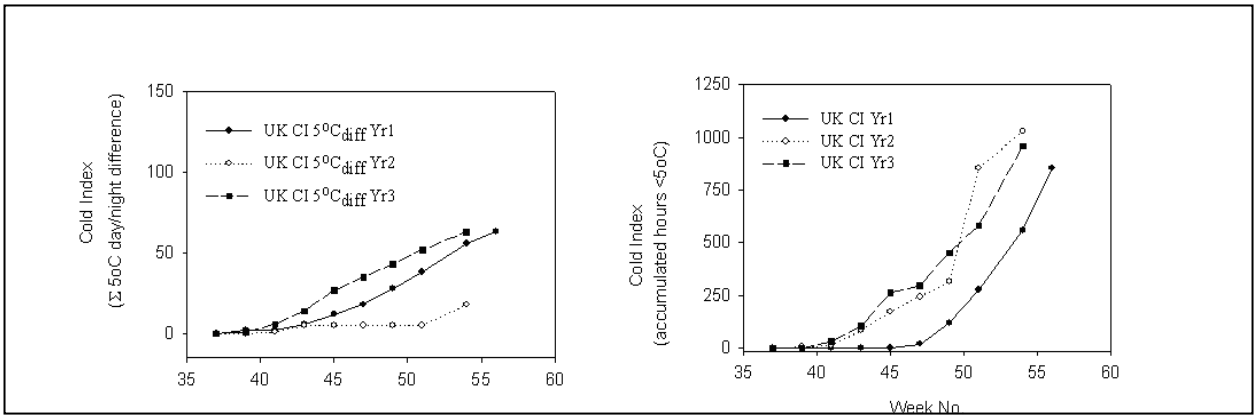

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Figure 3.8. Seasonal trends, observed over 3 years of study in the UK, in cold index calculated as: top panel - accumulated sum 5°C day/night thermal difference, bottom panel - accumulated sum <5°C hours.

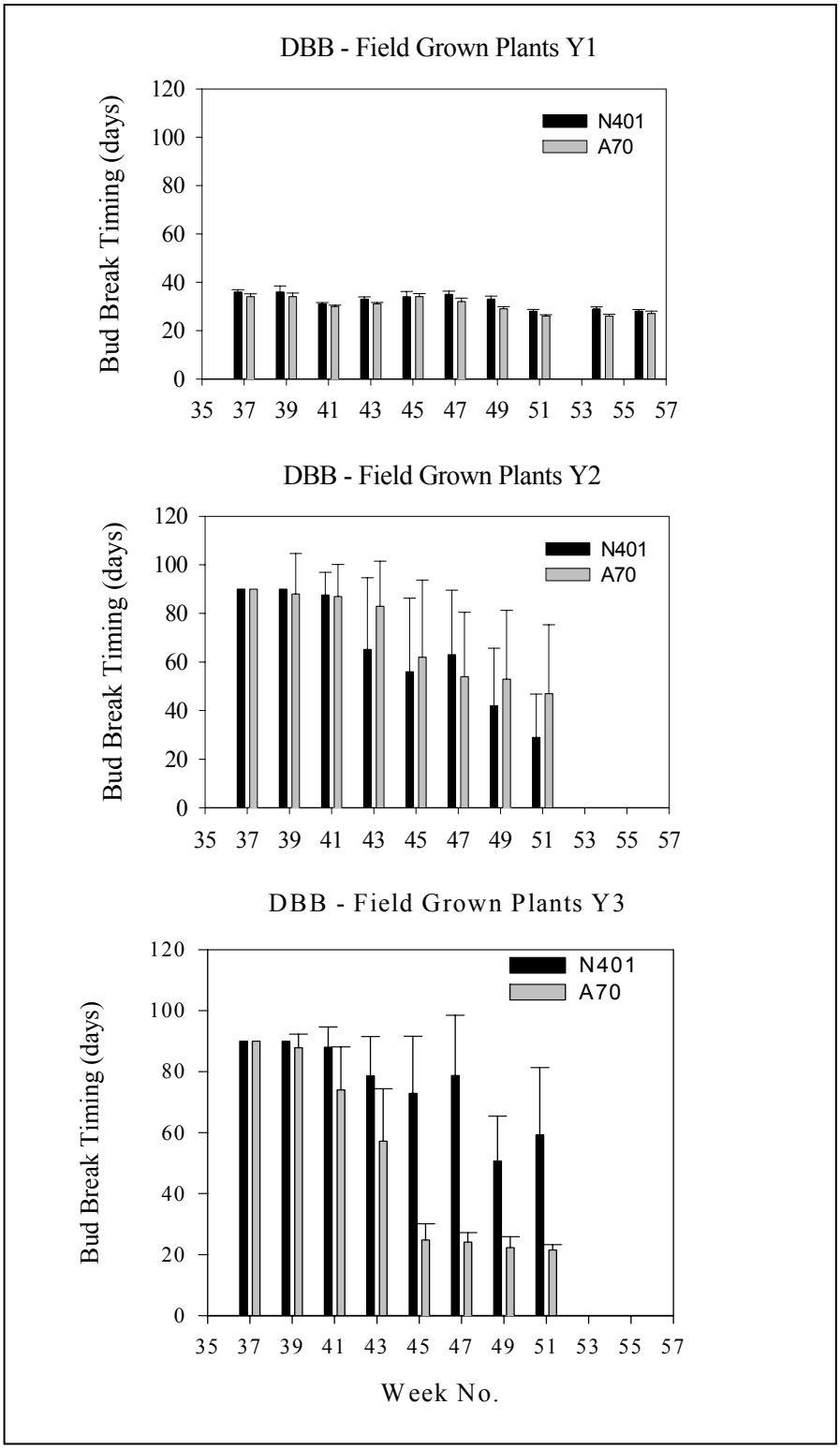


Figure 3.9. Physiological trends, relative to week of lifting, in the average days to bud break (DBB) response of the shoots, as assessed by growth under ideal conditions (*n* = 15), of standard Scots pine provenance (A70) and local (N401) provence grown in the UK subjected to ambient conditions. Upper panel – DBB year 1, middle panel – Dbb year 2, bottom panel – DBB year 3.

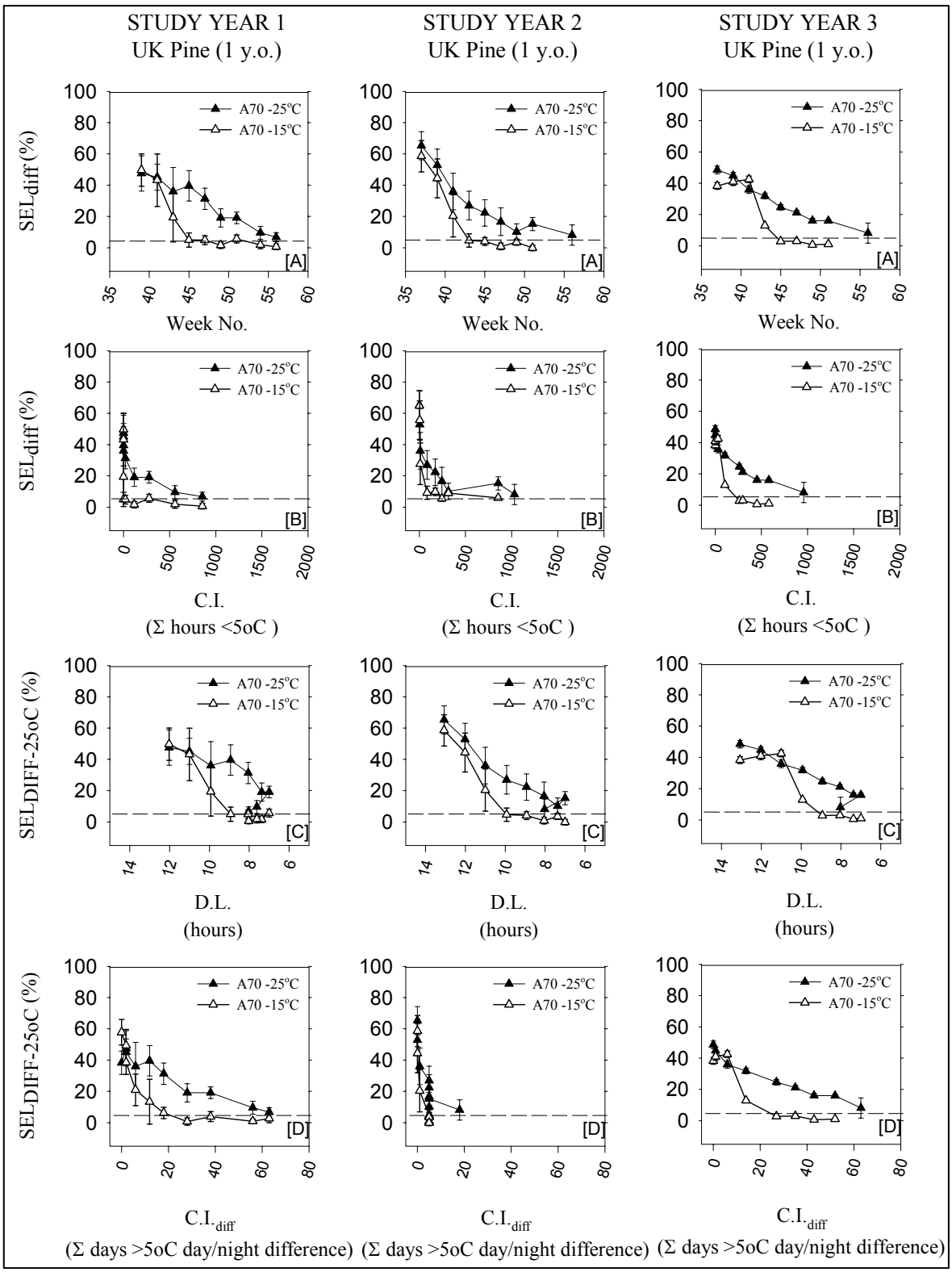


Figure 3.10. Seasonal physiological trends, with respect to year of experiment, in the average cold tolerance response of shoots, relative to week of lifting. The data description is varied by environmental cue, in the three lower panels.

field performance. Under UK conditions it is apparent that in the second year of study highly significant correlations were evident with second year field survival. The first year data has not been presented due to the confounding effect of opportunistic grazing of the field trial by sheep, which requires further in-depth analysis. In the third year significant correlations were observed at the end of the first growing season for native grown stock (N401) but no correlations were obtained for A70 seedlings as survival was observed to be 100%. However, this may alter when second year survival is assessed at the end of the 2005 growing season.

3.2 Field trials using beech

Cold Hardiness Assessments

Beech seedlings developed shoot frost tolerance in both the UK and Denmark, in the first year of experiments (Figure 3.11), although deeper levels of cold acclimation occurred in Denmark than the UK, as evidenced by SELdiff –25°C. Assuming a threshold of 10% for cold hardiness of broadleaf beech seedlings would be frost tolerant to –15°C by week 47, for all provenances and locations. No root acclimation was observed, tested at either –5°C or –2°C (data not shown). Overall the shoot cold hardiness development in beech seedlings grown outside was greater than in the growth room seedlings (cf. Figure 3.22). No provenance effects were observed at the UK or Danish sites (Figure 3.11). In the first year of study (Year 1) for all provenances a significant effect of lifting date was evident (Figure 3.11).

The seasonal differences in cold tolerance development for beech, grown under Danish and UK conditions, showed that (inter-) annual development of shoot cold tolerance, assessed after artificial freeze testing, occurred at both locations and for all provenances (Figure 3.12). However a -25°C tolerance threshold was reached later for UK grown material (and not at all in 2003). Within country comparisons between standard and local material revealed no significant shoot cold tolerance differences between provenances, irrespective of artificial freeze temperature (full data-set not shown). Much greater variation was observed in the standard (Danish) provenance (Bregentved) in the development of shoot cold tolerance under UK conditions (Figure 3.12), and fluctuations were particularly evident for test at -25°C.

The influence of seedling age upon the rate and level of cold tolerance development for beech, grown in Denmark, UK and the Netherlands was also investigated (Figure 3.13). Within Denmark the standard provenance showed that (inter-) annual development of shoot cold tolerance, assessed after artificial freeze testing varied with respect to seedling age, i.e. during the climatically colder second year 2-year-old seedlings developed deeper cold tolerance (Figure 3.13A). Furthermore, 2-year old seedlings were ahead of 1-year olds by approximately 2 weeks. The older seedlings reached the 10%-threshold for SELdiff-25 in week 46 and the 1-year old seedlings in week 48-49. Comparison within other countries showed age-effects for the UK (Figure 3.13B) and the Netherlands (Figure 3.13C). In particular the onset of cold tolerance was greatly advanced under UK conditions in material that had already been overwintered for one season.

Dormancy (DBB) Index

In Denmark the bare rooted beech seedlings entered dormancy around week 41 and was released, or at least back to the initial level by week no. 4 (Figure 3.14A). There were no clear physiological differences between the standard provenance ‘Bregentved’ and the local provenance ‘Gråsten’. Dormancy in beech was considerably deeper than in pines, and the ontogenetic pattern of dormancy development was later in the UK provenance than the standard Danish provenance (Figure 3.14B). Comparison of the standard provenance grown at these two sites showed that UK grown seedlings developed deeper dormancy levels earlier than those under Danish conditions (Figure 3.14A,B), but loss of dormancy did not differ significantly.

Development of bud dormancy in Danish grown stock was earlier and deeper in 2-year old than in 1-year old seedlings, though both reached a maximum level of approximately 60 DBB in week 47, in year 2 (Figure 3.15A). In UK grown material high levels of dormancy were evident from the start of the trial in the second year, with no noticeable effect of provenance. At both sites patterns similar to the previous year were

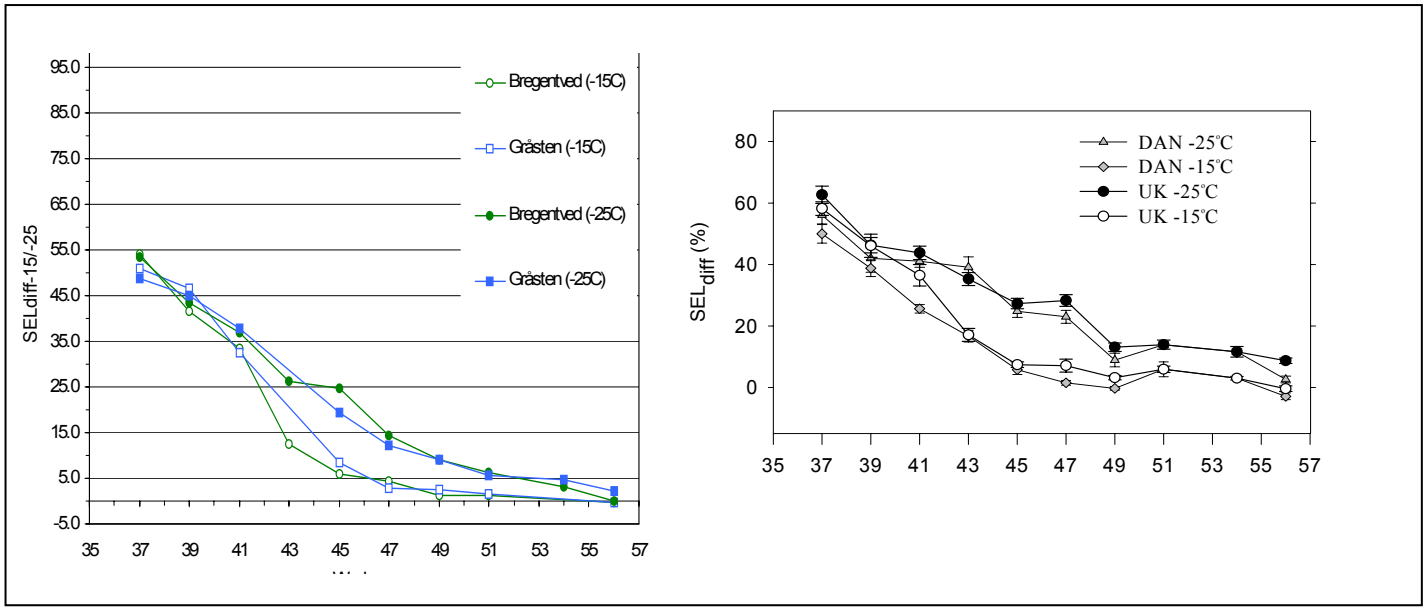


Figure 3.11. Provenance effects on seasonal physiological trends (observed in Year 1) in the average cold tolerance response of the shoots, as assessed by electrolyte leakage after artificial freezing (*n* = 15), of standard and local Beech provenances in A] Denmark and B] the UK, relative to week of lifting

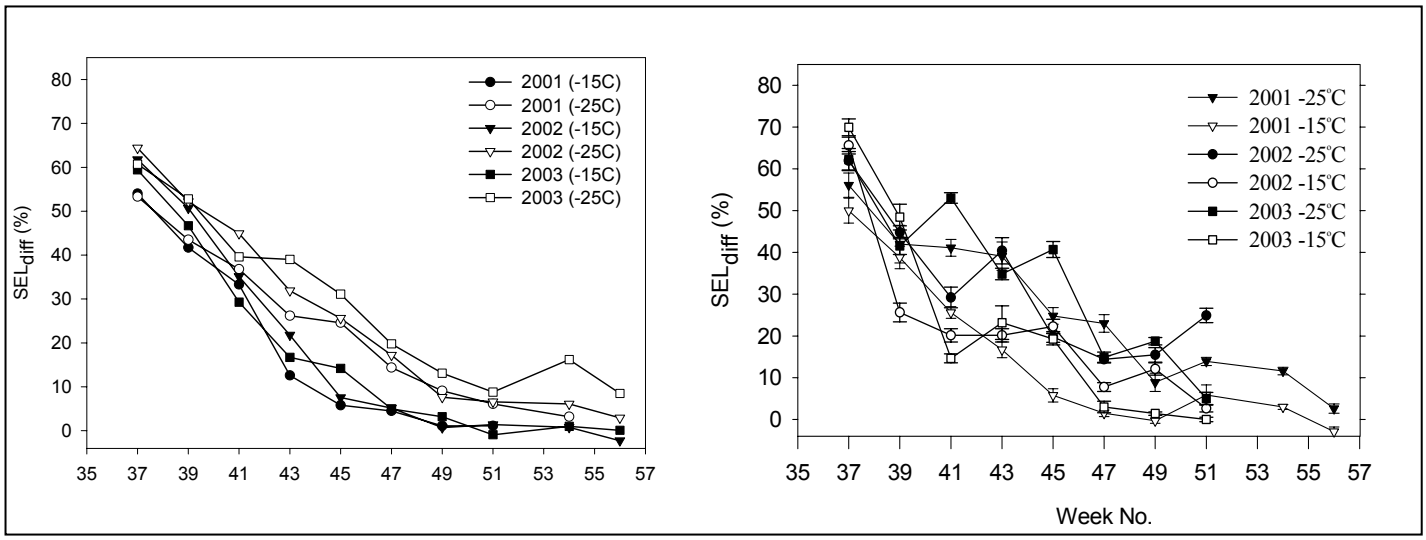


Figure 3.12. Intercomparison of seasonal physiological trends observed across all years in the average cold tolerance response of the shoots, as assessed by electrolyte leakage after artificial freezing (*n* = 15), of standard Beech provenance 'Bregentved' in A] Denmark and B] the UK, relative to week of lifting.

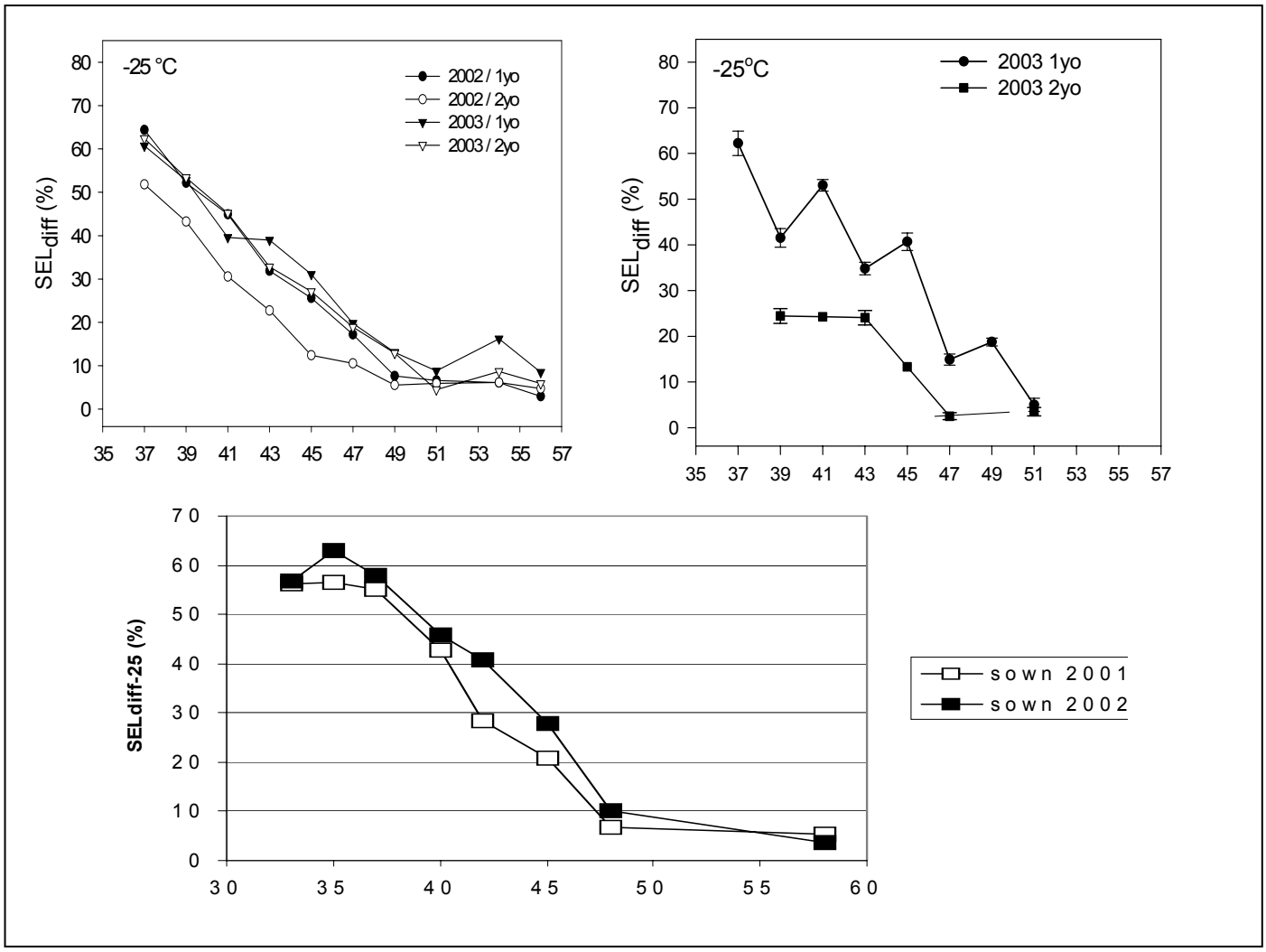


Figure 3.13. Intercomparison of seasonal physiological trends observed with respect to seedling age in the average cold tolerance response of the shoots, as assessed by electrolyte leakage after artificial freezing (*n* = 15), of standard Beech provenance 'Bregentved' in A] Denmark (Y2 & Y3), B] the UK (Y3), and C] the Netherlands (Y2), relative to week of lifting.

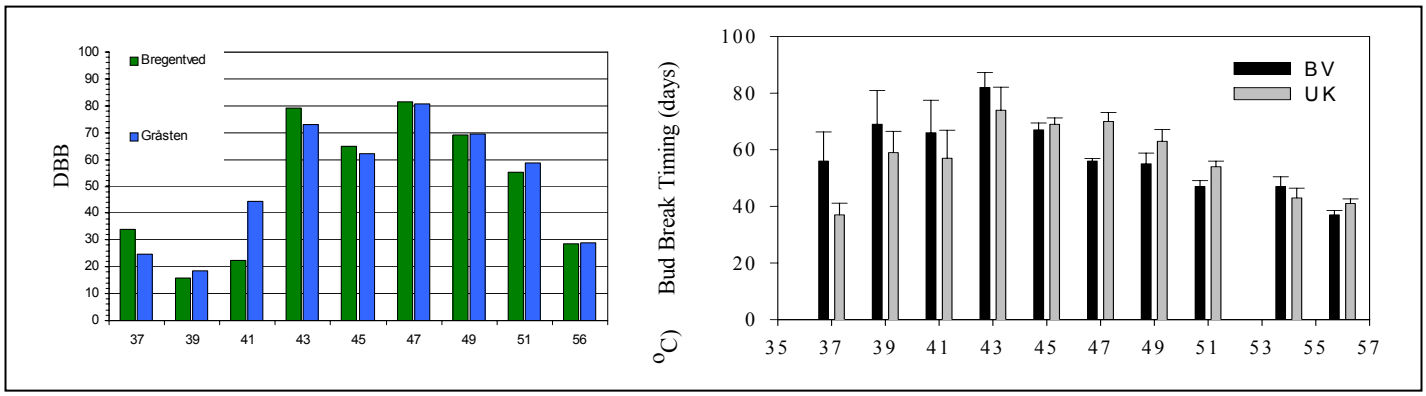


Figure 3.14. Intercomparison of seasonal physiological trends in dormancy observed with respect to provenance, in the first year of study, as assessed by days to budburst (*n* = 15), of standard Beech provenance 'Bregentved' and local provenance in A] Denmark, and B] the UK, relative to week of lifting.

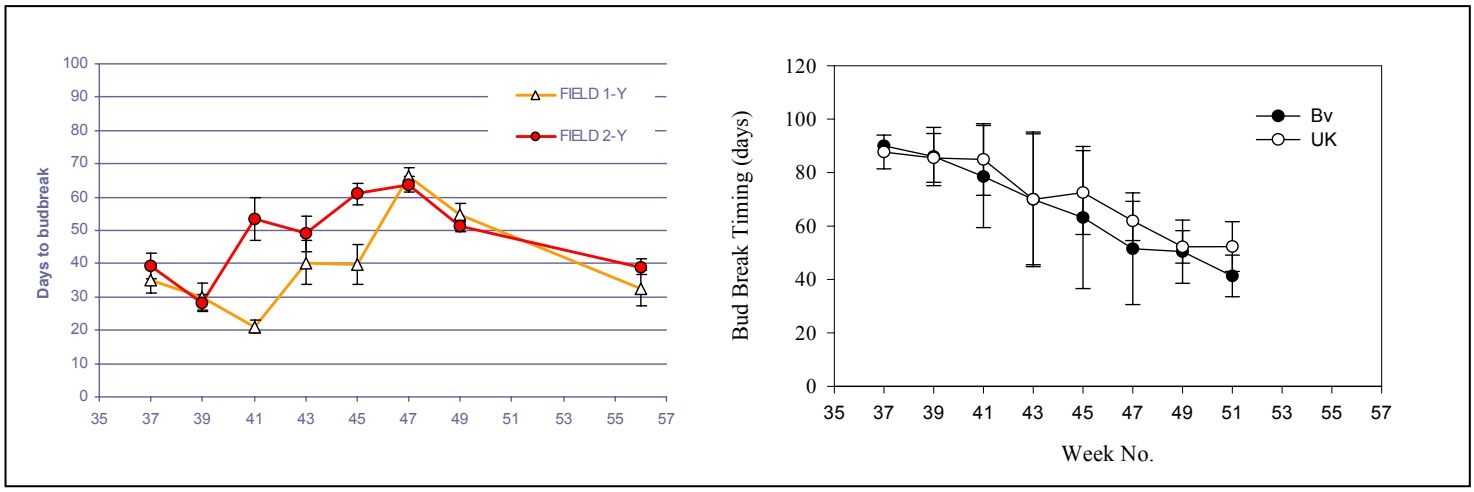


Figure 3.15. Intercomparison of seasonal physiological trends in dormancy observed with respect to provenance, in the second year of study, as assessed by days to budburst (*n* = 15), of standard Beech provenance 'Bregentved' and local provenance in A] Denmark, and B] the UK, relative to week of lifting.

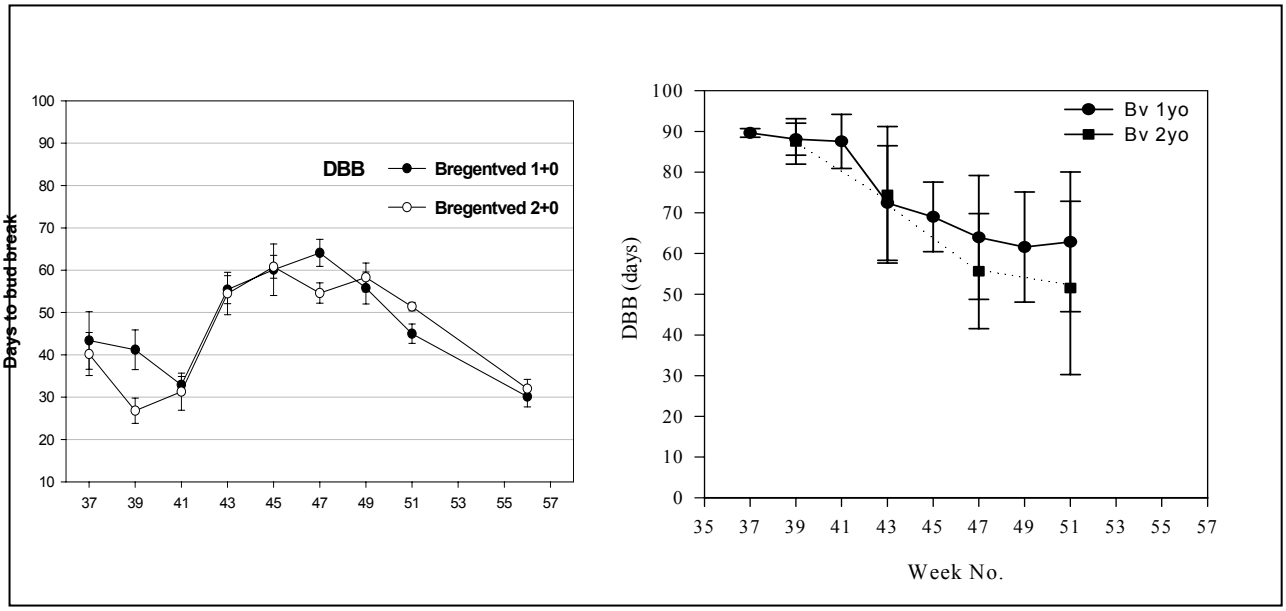


Figure 3.16. Intercomparison of seasonal physiological trends in dormancy observed with respect to seedling age, in the third year of study, as assessed by days to budburst (*n* = 15), of standard Beech provenance 'Bregentved' in A] Denmark, and B] the UK, relative to week of lifting.

evident in the third trial (Figure 3.16), with the exception that age-related effects were not evident for Danish grown stock.

Field Performance
First- and second-year field performance of one-year old beech seedlings lifted in 2001 was recorded in January 2003 and January 2004 respectively (data not shown). First-year height increment increased with later lifting dates and tended to stabilise from week 45 but showed a rather uncertain development later in the season (from week 49). The same (transient) decline was seen in stem diameter growth. This growth depression, however, was no longer evident after two growing season. The results therefore suggest 2001 grown one-year old beech seedling were storable from week 45. First-year height and diameter growth of one-year old beech seedlings lifted in show that seedlings were not completely storable until week 47. In the same year safe lifting of two-year old seedlings was possible from week 41 with respect to optimizing height growth, while safe lifting with respect to stem diameter growth was not possible until week 45. The effect of earlier cold acclimation in two-year old beech seedlings, as evidenced from SEL tests, therefore accurately reflected field performance (i.e. improved growth of early lifted seedlings). It is also clear that the negative effect of early lifting was maintained for at least two years even though growth performance levelled out with time.

3.3 Climate room trials using pine
First Year Experiments
Pine seedlings from all controlled environment climate regimes showed development of root frost tolerance assessed at -5°C, and shoot frost tolerance as assessed by SELdiff -15°C, but this was not evident at the SELdiff -25°C assessment temperature (Figure 3.17). This indicates that plants had not developed hardiness to this level under the controlled environment conditions. Shoot frost hardiness assessed at -15°C indicated that there was a lower level (lack of continued) hardiness induction in Climate 1 (Control) seedlings over the last four weeks of the trial.
All experimental plant types had a more or less distinct dormancy period, even the Climate 1 (control) seedlings. Under controlled conditions declines in both temperature and daylength led to a decrease in dormancy levels relative to the control.

Second Year Experiments
Pine seedlings from the controlled environment climate regimes in the second year of study showed no significant development of root frost tolerance, as assessed by RELdiff -5°C (Figure 3.18). However, root based assessments did show effects of SCOLD and SWARM treatments. Both freeze temperature assessments of shoot frost tolerance, by SELdiff –15°C and SELdiff -25°C, showed seasonal patterns of cold tolerance development for seedlings treated in the declining environment (Figure 3.18). Clear differences in shoot cold hardiness were evident, in comparison with Climate 1 (control) seedlings, over the last four weeks of the trial. There was no evidence of changes in shoot physiology resulting from cold or warm ‘shock’ periods (SCOLD & SWARM when compared with the seedlings maintained under the DECLINE climate. The number of days to bud burst, which is an expression of the level of dormancy in the seedling, showed a decline from maximal levels (90 days) under the declining conditions (Figure 3.19) in year 2. The analysis protocol using a maximum (unburst) threshold of 90 days was been adopted (after Heide 1993a, b).
All experimental plant types had a distinct dormancy period with the exception of plants in the control climate treatment, which did not develop any (release from) dormancy during the second year (Figure 3.20). Under controlled conditions declining temperature and daylength did lead to a decrease in dormancy levels, which was not evident for control climate treatment material (Figure 3.20). This suggests the 5°C day/night temperature difference is ‘critical’ in (pseudo) dormancy development in this species.

Novel DBB analysis
Kaplan-Meier ‘survival distribution function’ analysis was performed for UK grown material (including material from climate room trials pine, field trials pine and beech) i.e.

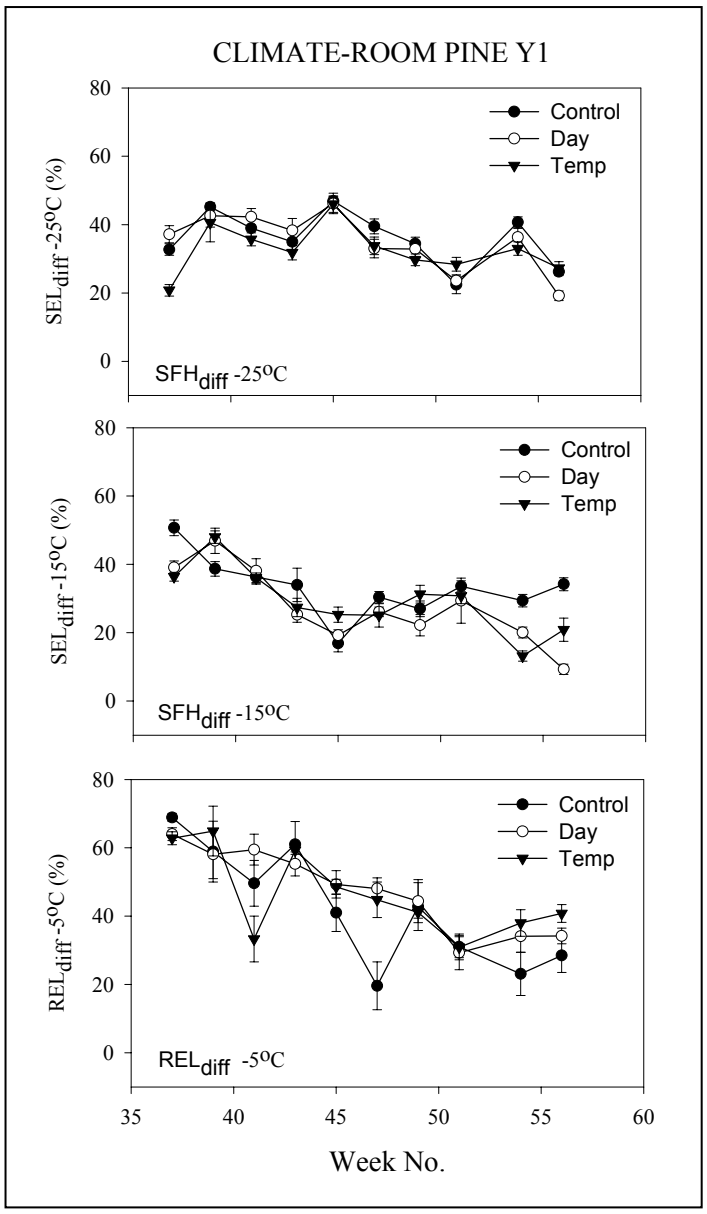


Figure 3.17. Physiological trends, observed in Year 1, relative to week of lifting in the average cold tolerance response of the shoots, as assessed by electrolyte leakage after artificial freezing (*n* = 15), of standard Scots pine provenance grown in the UK and subjected to controlled environmental conditions in a growth chamber.
Upper panel – shoot electrolyte leakage (SEL_{diff}) of standard A70 provenance tested at –25°C, middle panel - shoot electrolyte leakage (SEL_{diff}) of standard A70 provenance tested after –15°C, bottom panels - post-freeze root electrolyte leakage (REL_{diff}) tested after –5°C.

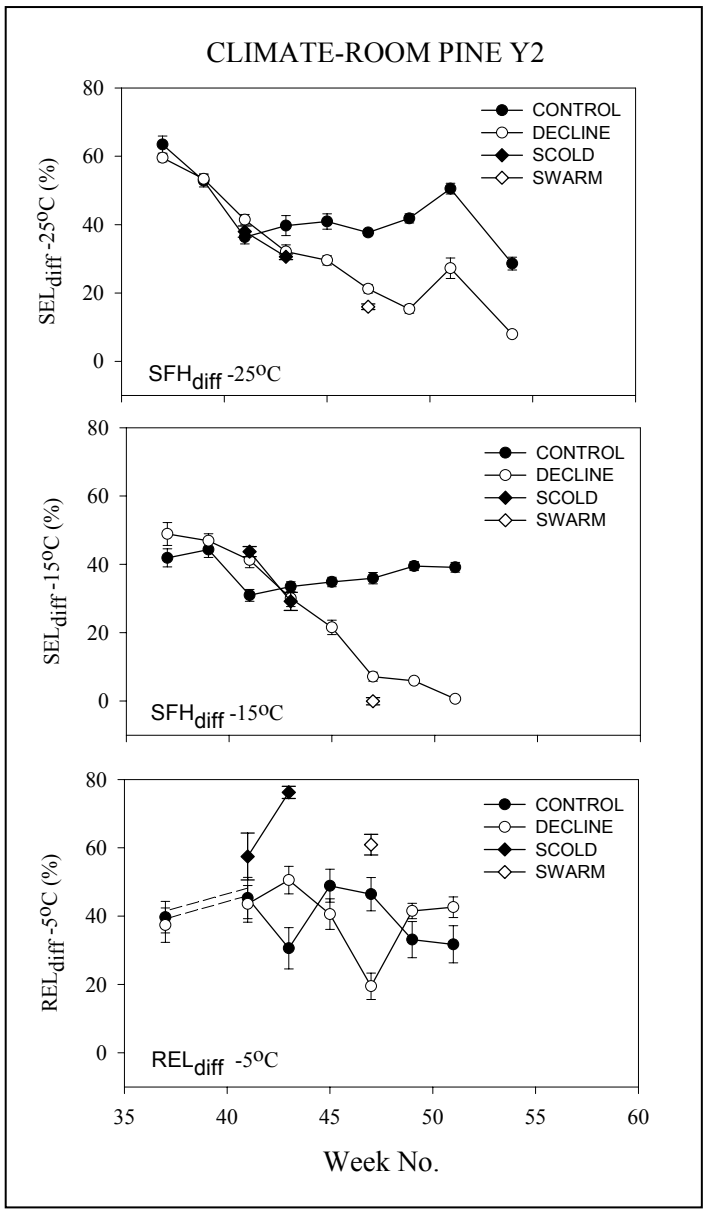


Figure 3.18. Physiological trends, observed in Year 2, relative to week of lifting in the average cold tolerance response of the shoots, as assessed by electrolyte leakage after artificial freezing (*n* = 15), of standard Scots pine provenance grown in the UK and subjected to controlled environmental conditions in a growth chamber.
Upper panel – shoot electrolyte leakage (SEL_{diff}) of standard A70 provenance tested at –25°C, middle panel - shoot electrolyte leakage (SEL_{diff}) of standard A70 provenance tested after –15°C, bottom panels - post-freeze root electrolyte leakage (REL_{diff}) tested after –5°C

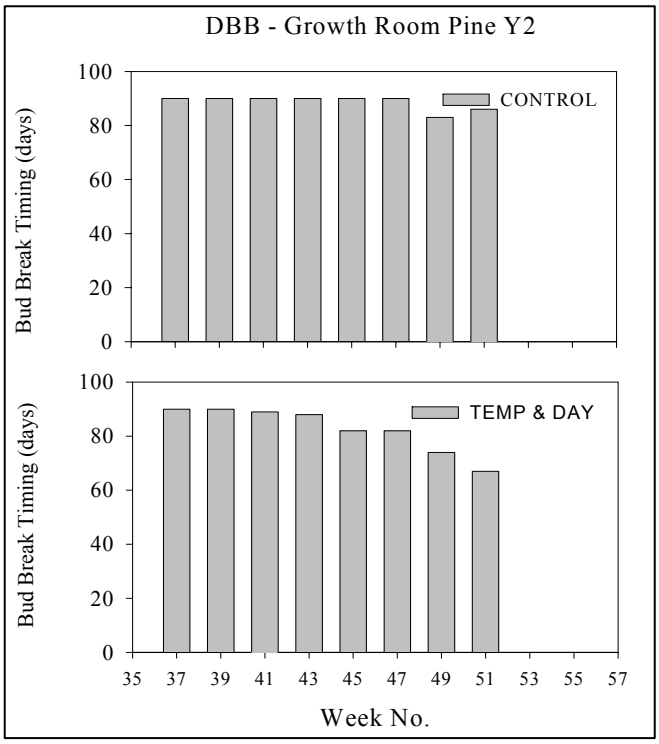


Figure 3.19. Physiological trends, observed in Year 2, relative to week of lifting in the average days to bud break (DBB) response of the shoots, as assessed by growth under ideal conditions (*n* = 15), of standard Scots pine provenance grown in the UK and subjected to controlled environmental conditions in a growth chamber.
Upper panel – DBB of standard A70 provenance after control conditions, lower panel - DBB of standard A70 provenance after conditions with declining temperature and daylength.

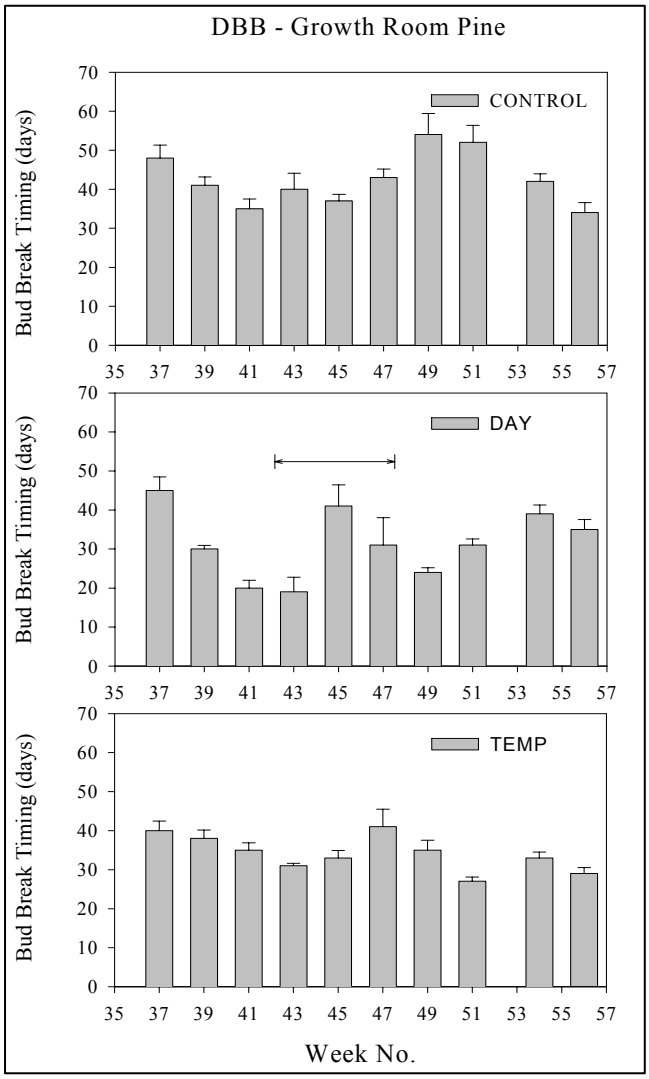


Figure 3.20. Physiological trends, observed in Year 1, relative to week of lifting in the average days to bud break (DBB) response of the shoots, as assessed by growth under ideal conditions (*n* = 15), of standard Scots pine provenance grown in the UK and subjected to controlled environmental conditions in a growth chamber.
Upper panel – DBB of standard A70 provenance after control conditions, middle panel - DBB of standard A70 provenance after conditions with declining daylength, bottom panels - DBB of standard A70 provenance after conditions with declining temperature.

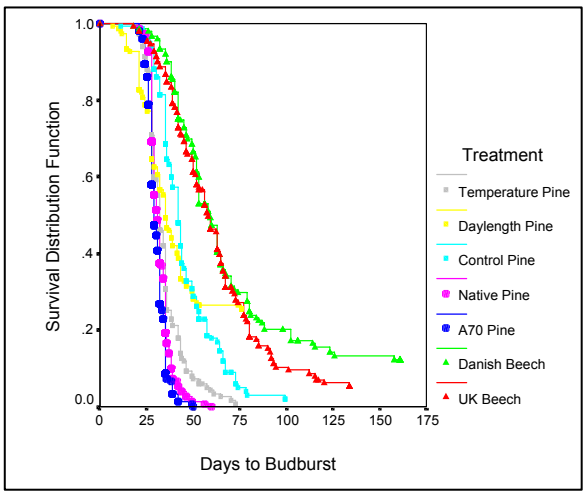


Figure 3.21. Kaplan-Meier 'survival time analysis' curves showing the survival distribution function for the seven treatments (from climate room trials using pine and field trials with both beech and pine). Censored points are shown at their minimum possible value (i.e. the maximum days to budburst recorded for that treatment on the lift date from where the censored data point originates).

for seven treatments (Figure 3.21). We look for gaps in these curves in a horizontal or vertical direction. A vertical gap means that at a specific time point, one group had a greater fraction of subject 'events'. A horizontal gap means that it took longer for one group to experience a certain fraction of 'events'. Analysis based on this method shows that the seven treatments were shown to be significantly different from each other using both Wilcoxon rank test ($p<0.0001$) and a likelihood ratio test ($p<0.0001$). The ten lift dates were also shown to be significantly different from each other (Wilcoxon rank test: $P<0.0001$, likelihood ratio test: $P<0.01$) with lifts five and six having the deepest dormancy (data not shown).

3.4 climate room trials using beech

Cold Hardiness development in controlled environment conditions.

The standard beech provenance 'Bregentved' (Czech origin, Danish seed source) exhibited increased cold tolerance during autumn, for controlled environment grown seedlings under all imposed climate regimes, as evidenced by the physiological assessment of SELdiff-15 and SELdiff-25 (Figure 3.22). It should be noted that due to extremely high conductivity of the deionised water used for electrolyte leakage measurements in week no. 37, conductivity results from this week should not be considered reliable. The week 37 data is, however, presented in all figures. The results show that shoots became frost tolerant to –15°C first. Shoots continued to develop tolerance (cold hardiness) to temperatures lower than -15°C, but did not reach a threshold tolerance to -25°C, i.e. this freeze test temperature caused >10% leakage of test samples (Figure 3.22, lower panel). Seedlings in the control climates (long day/high temperature) also attained some cold hardiness (Figure 3.22, top panel) though both the rate of development and level of maximal tolerance were reduced. Therefore, long days and high temperature did not prevent the onset and development of cold hardiness and dormancy in control treatment plants, even when DL had been extended by two hours in the second trial season (2002-2003).

No development of root frost tolerance to –5°C was observed in beech, irrespective of treatment or location i.e. controlled or natural (outdoor) growth conditions and therefore assessments of root hardiness were discontinued after the first year (data not shown).

Dormancy development in controlled environment conditions.

Beech seedlings from all (three) climate regimes, in year 1, developed almost equal levels of dormancy, as evidenced by the number of days to terminal bud break under favourable growth conditions. Climate 3 seedlings appeared to reach greater levels of maximum dormancy, which occurred in week 45, two to four weeks before maximal dormancy development in the other two climates (Figure 3.23). In year 2 plants from both experimental treatments exhibited similar dormancy periods though high dormancy levels developed 2 weeks earlier (week 43) in control environment seedlings whilst a slow step-wise loss of bud dormancy was observed in seedlings grown under declining conditions, after week 47.

The general conclusions from experiments in the second year with short-period cold or warm shocks is that abrupt short-term changes in climatic conditions do not significantly interfere with dormancy and frost tolerance development (Figure 3.24).

3.5 Storability trials using pine

Vitality

The electrolyte leakage for unfrozen shoots (SEL) showed that the cell integrity of the seedlings that were put into storage in week 40 was damaged when the seedlings were taken out of the storage in week 17 (Figure 3.25, upper panels). The damage to the shoots was obvious already in week 7 for the 1-year-old seedlings and for the 2-year-old A70 seedlings. For these seedlings there was also a rise in the electrolyte leakage of the unfrozen roots (REL) in week 17 (Figure 3.26, upper panels and right mid-panel). This indicates that the cells of the roots were damaged as well. Storage later in season, in week 44 and 48 did not cause an increased electrolyte leakage for Sollerön and Åmsele seedlings (Figure 3.25 and 3.26 mid and lower panels). However, the 2-year-old A70 seedlings stored in week 44 and 48 showed increased shoot electrolyte

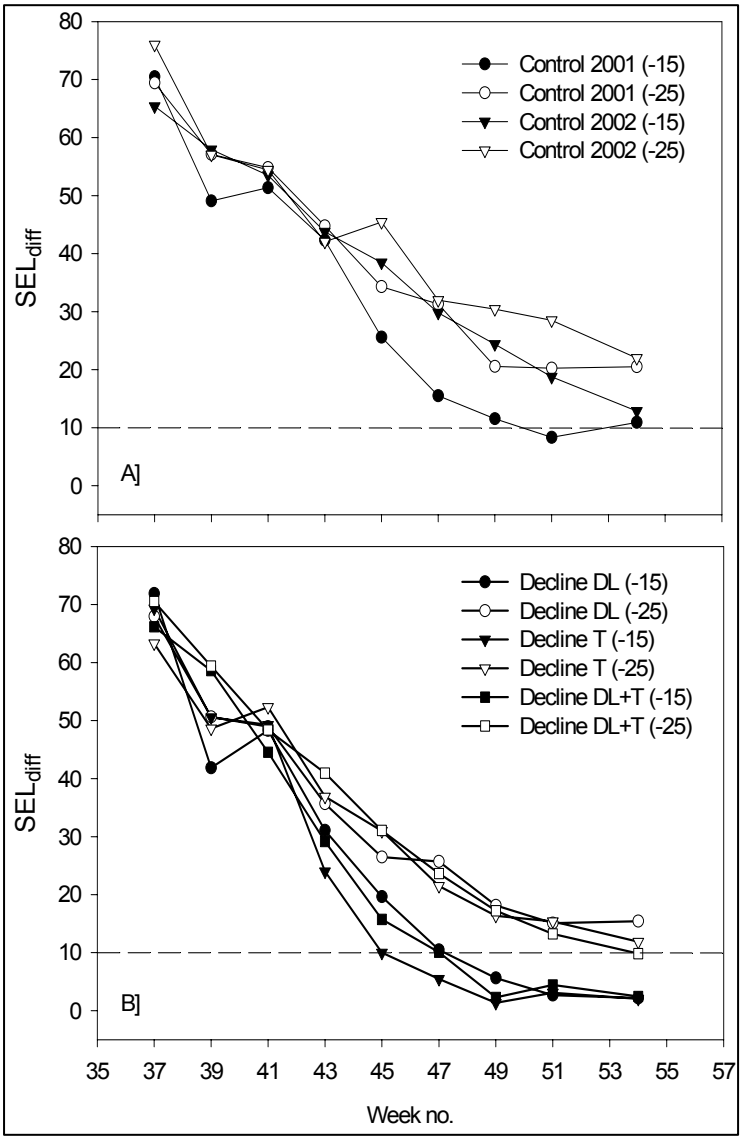


Figure 3.22. Cold hardiness as assessed by shoot electrolyte leakage (SEL_{diff}) in relation to temperature and daylength manipulations during two controlled-environment studies, conducted in 2001 and 2002. A] cold hardiness development, assessed as ability to withstand shoot freezing to -15°C or -25°C, after growth under control (ideal) conditions. B] cold hardiness development, assessed as ability to withstand shoot freezing to -15°C or -25°C, after growth under manipulated environmental conditions. Legend: DL= daylength, T = temperature.

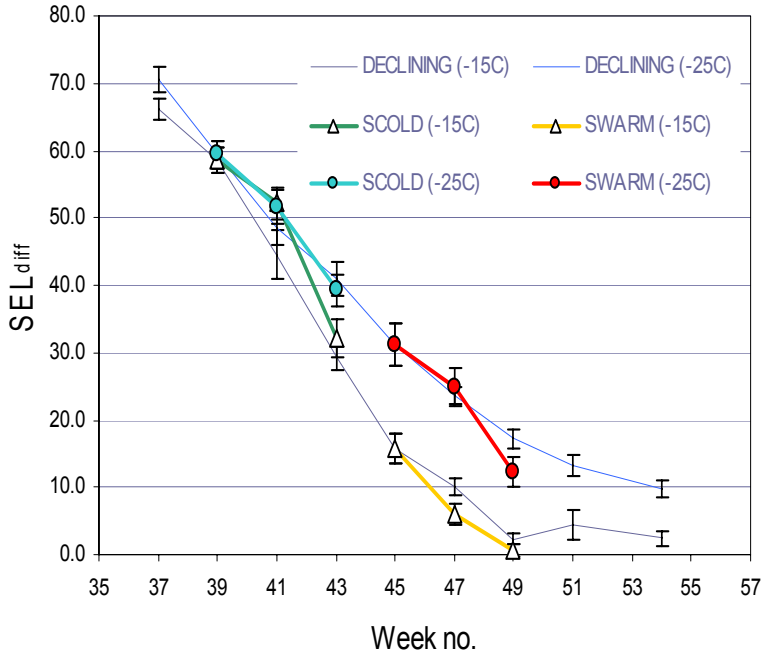


Figure 3.24. Cold hardiness as assessed by shoot electrolyte leakage (SEL_{diff}) in relation to temperature and daylength manipulations during the controlled-environment study conducted in 2002. Cold hardiness development assessed as ability to withstand artificial shoot freezing to -15°C or -25°C, after growth under declining daylength and temperature, and the influence of short term removal to a warm (5°C higher than declining treatment) or cold (5°C lower than declining treatment) shock.

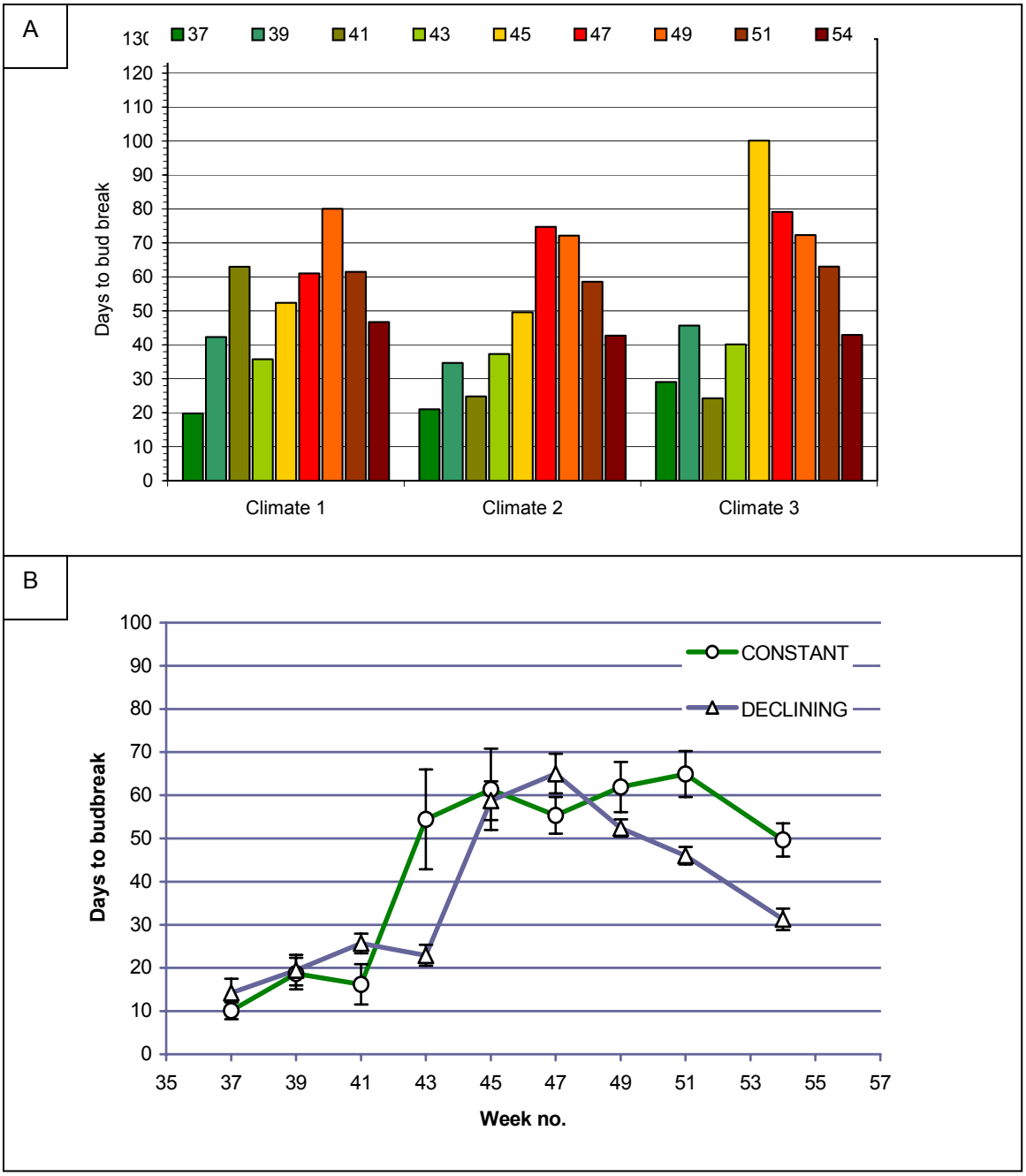


Figure 3.23.

A. Dormancy as assessed by days to budbreak (DBB) in relation to temperature and daylength manipulations during two controlled-environment studies, conducted in 2001 and 2002. Top panel is DBB after growth under one of three controlled conditions in 2001: Climate 1 (control) daylength=15h, temperatures T_{day}=15°C and T_{night}=13°C, Climate 2 (daylength) as climate 1, but DL reduced by 1h every two weeks until 8.5 hours, Climate 3 (temperature) as climate 1, but T_{day} and T_{night} reduced by 2°C every two weeks until +1°C/+1°C.

B. Dormancy levels, assessed under one of two controlled conditions in 2002: Constant (control) daylength=15h, temperatures T_{day}=15°C and T_{night}=13°C, or Declining, as climate 1, but DL reduced by 1h every two weeks until 8.5 hours and T_{day} and T_{night} reduced by 2°C every two weeks until +1°C/+1°C.

leakage at the end of the storage period. Seedling mortality assessed in the regrowth tests after frozen storage is presented in Figure 3.27. Even though the 2-year-old A70 seedlings were not planted in regrowth tests due to lack of seedlings does the general picture of mortality (Figure 3.27) agree with the picture of vitality shown in Figure 3.25. Seedlings with survival above 85 % had all, except one treatment, SEL values below 13 % (Figure 3.28) and only one SEL value above 13 % corresponded to survival above 85 %.

Shoot and root freezing tolerance
The roots did not change in freezing tolerance during the storage (not shown). However, the shoots displayed a dehardening (Figure 3.29). The freezing tolerance is only shown for seedlings that were considered vital i.e. had SEL values below 13 %. The SELdiff-25 values increased from ranging between 0 to 5 at the start of storage to range between 5 and 15 at the end of the storage period.

Storability measurements
The seedlings shoot freezing tolerance is commonly used as a measurement of storability. In Sweden the freezing tolerance measured with the freeze induced electrolyte leakage method is often used. The assessment is commonly made on 30 seedlings of which 15 are tested for baseline leakage and 15 frozen to -25 °C. The 15 seedlings are tested as 5 replicates, each consisting of three seedlings. To be considered storable all 5 samples have to have a SELdiff-25 value below 5.

In this study several of the seedlings were not storable according to this standard (Table 3.1). At the first storage date in week 40 only the 2-year-old Åmsele seedlings had a SELdiff-25 value below 5. In spite of this these seedlings showed poor vitality at the end of storage (Figure 3.30). The probable cause to the poor vitality is that the roots of these seedlings were not tolerant to the temperature in the storage. The RELdiff-5 value when the seedlings were put into storage was 17 (Table 3.1). This means that the freezing to -5 °C, which also was the actual temperature in the storage, caused substantial damage to the roots. The A70 seedlings never reached SELdiff-25 values below 5, but were close in week 44 when the 1-year-old seedlings had a SELdiff-25 value of 5.7. The vitality checked as root growth capacity at the end of the storage period also shows that the A70 seedlings had reduced or no capacity to induce new root growth (Figure 3.30). The 1-year-old Sollerön seedlings reached the storability limit in week 44 (Table 3.1) and root growth capacity at the end of storage period was high (Figure 3.30). However, the SELdiff-25 value for these seedlings had risen above 5 in week 48 and they should therefore not be considered storable. The standard deviation (Table 3.1) implies that this is not a true dehardening and the root growth capacity that was measured after storage shows that the seedlings actually still were storable (Figure 3.30).

Table 3.1. Shoot ($SEL_{diff-25}$) and root (REL_{diff-5}) freezing tolerance of 1- and 2-year-old Scots pine seedlings of three provenances in week 40, 44 and 48, 2003. Standard deviation in brackets. Shoot N = 15, Root N = 5

	A70		Sollerön		Åmsele	
	1-year	2-year	1-year	2-year	1-year	2-year
$SEL_{diff-25}$						
Week 40	35.5 (*13.8*)	14.9 (*12.0*)	28.4 (*3.7*)	7.0 (*8.2*)	15.4 (*8.6*)	2.8 (*3.1*)
Week 44	5.7 (*7.9*)	14.2 (*12.4*)	4.0 (*6.7*)	0.0 (*1.3*)	3.9 (*2.9*)	0.9 (*1.8*)
Week 48	14.3 (*6.7*)	17.6 (*19.4*)	6.5 (*2.7*)	2.6 (*8.8*)	3.5 (*1.9*)	2.0 (*1.5*)
REL_{diff-5}						
Week 40	45.8 (*16.8*)	20.7 (*7.3*)	38.0 (*5.3*)	10.5 (*3.8*)	36.7 (*7.9*)	17.0 (*6.1*)
Week 44	11.3 (*5.5*)	1.2 (*3.7*)	3.3 (*3.9*)	0.1 (*2.5*)	2.0 (*3.1*)	2.3 (*4.0*)
Week 48	5.0 (*8.0*)	-4.1 (*7.7*)	2.7 (*2.1*)	-0.2 (*4.6*)	3.9 (*1.7*)	-3.0 (*3.6*)

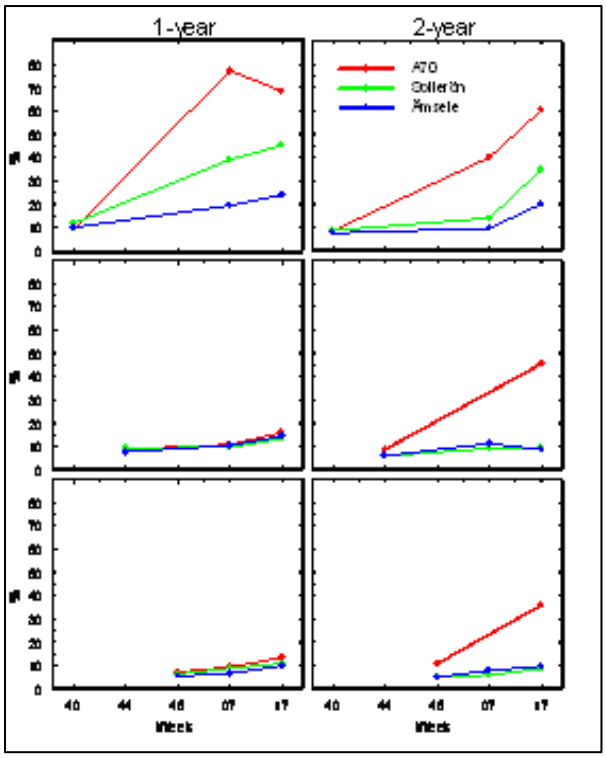


Figure 3.25. Shoot electrolyte leakage (%) of 1- and 2-year-old Scots pine seedlings of three provenances (Table 2.2) in 2002 and 2003. N = 15

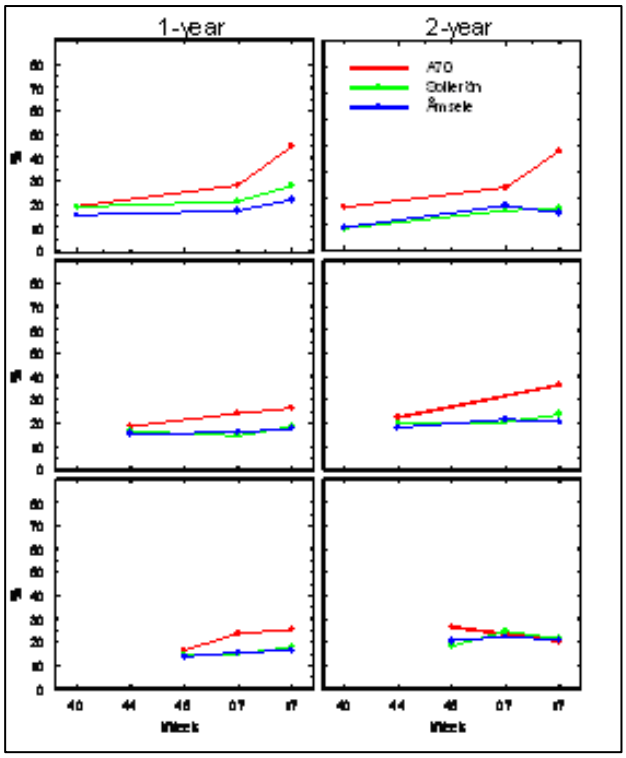


Figure 3.26. Root electrolyte leakage (%) of 1-, 2- and 3-year-old Scots pine seedlings of three provenances (Table 2.2) in 2002 and 2003. N = 5

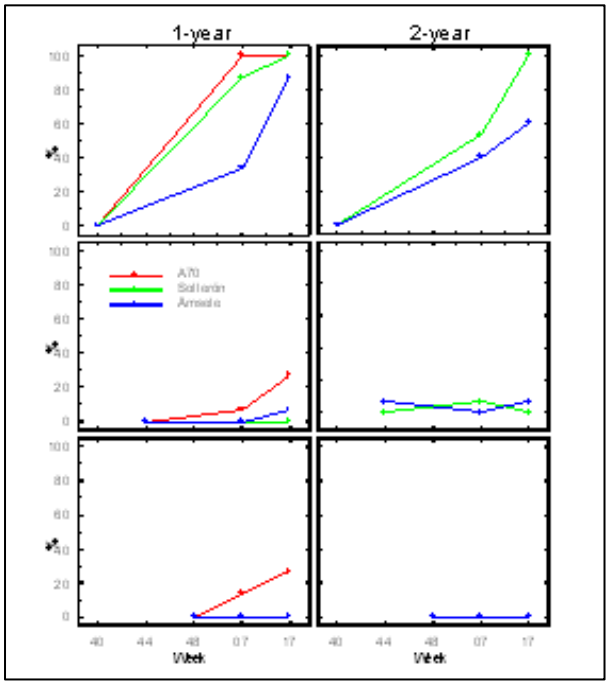


Figure 3.27. Seedling mortality (%) of 1- and 2-year-old Scots pine seedlings of three provenances after frozen storage. N = 15

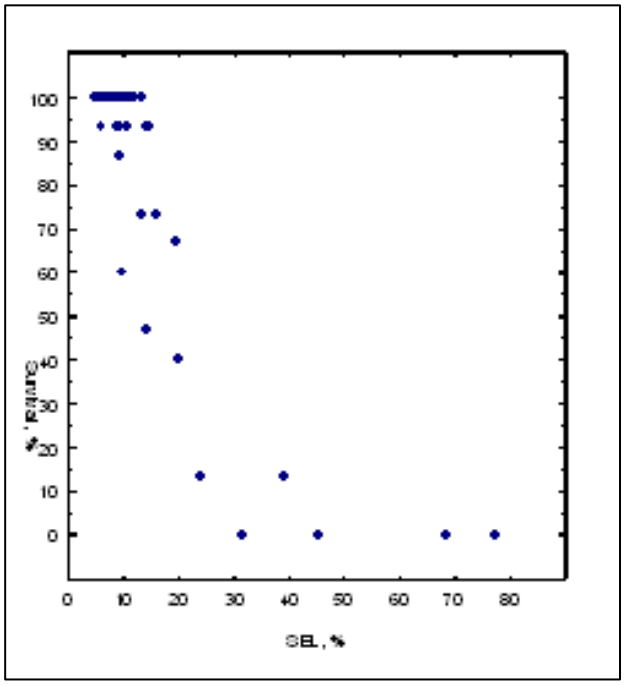


Figure 3.28. Shoot electrolyte leakage, SEL (%) of 1- and 2-year-old Scots pine seedlings of three provenances versus survival (%). N = 15

3.6 Provenance comparison for pine

Shoot and root freezing tolerance of the standard provenances

The baseline leakage (control values) for shoots (Figure 3.31) and roots (Figure 3.32) showed normal values throughout the whole testing periods. This indicates that the seedlings were undamaged when they were taken in for determination of shoot and root freezing tolerance

The shoot electrolyte leakage of 2-year-old A70 seedlings in late 2003 is somewhat higher, though not significantly, than for the two other provenances. The highest level of shoot electrolyte leakage for the 2-year-old A70´s in 2003 (week 46 - 12.9 %) is a normal value and not an indication of damage.

Even though some of the provenances that were used in this study are not adapted to the climate in Garpenberg the Scots pine seedlings, of all provenances and ages, developed shoot (Figure 3.33) and root (Figure 3.34) freezing tolerance. There was a general increase in shoot freezing tolerance from week 36 to week 42/44 at which time the shoot freezing tolerance had reached a stable level. Roots increased in freezing tolerance during autumn and reached a stable level in week 44/46. There was a tendency that the 1-year-old seedlings were less shoot and root freezing tolerant early in autumn compared to the 2- and 3-year old seedlings. For the shoots, the age effect on freezing tolerance was clearly shown only in 2003 with the 2- and 3-year-old seedlings being more freezing tolerant than the 1-year-old seedlings early in season. In 2002, only the A70 seedlings showed difference in freezing tolerance between seedling ages.

Provenance effects for both shoot and root freezing tolerance were also found. The northern provenances achieved freezing tolerance earlier than the southern provenances. Moreover the British provenance A70 did not reach the same level of freezing tolerance as the more northern, Swedish, provenances. There was a tendency that the A70 seedlings lost shoot freezing tolerance in late autumn/early winter.

The standard deviations (SD) of the SELdiff and RELdiff values in this study were generally high. Significant differences between treatments could therefore only be established in some cases (all above 18 %). Early in season SD was normally around 40 percent. In late autumn, the deviation was around 100 % but could also be several 1000 %.

Shoot and root freezing tolerance - commercial seedlings

The baseline leakage (control treatment 2 °C) of the commercially grown seedlings showed that they were undamaged at the arrival to Garpenberg (Figure 3.35 A and B). Development of freezing tolerance in these seedlings showed the same picture (Figure 3.35C and D) as the standard provenances did (Figure 3.33 and 3.34). The shoots developed full freezing tolerance earlier than the roots and the more southern provenance developed full freezing tolerance later than the northern.

Bud dormancy – standard provenances

Several of the 1-year old seedlings had not set bud when they were taken into the greenhouse for bud break tests (Table 3.2), whereas all the 2- and 3-year old seedlings had formed buds already in week 36. Almost none of the 1-year-old A70 seedlings (Table 3.2) had formed buds at the start of the bud break tests and it took up to two weeks (Figure 3.36) for these seedlings to form an apical bud when taken into the growth promoting climate in the greenhouse. The 1-year-old Åmsele seedlings, however, had formed buds in week 40 (Table 3.2).

The number of days to bud burst, which is an expression of the level of dormancy in the seedling, showed a decline from early autumn to early winter (Figure 3.36). However, the 1-year-old seedlings, irrespective of provenance, never showed a true dormant stage. The A70 seedlings never displayed any rest/dormancy at all. The number of days to bud burst was almost the same through the whole sampling period for these

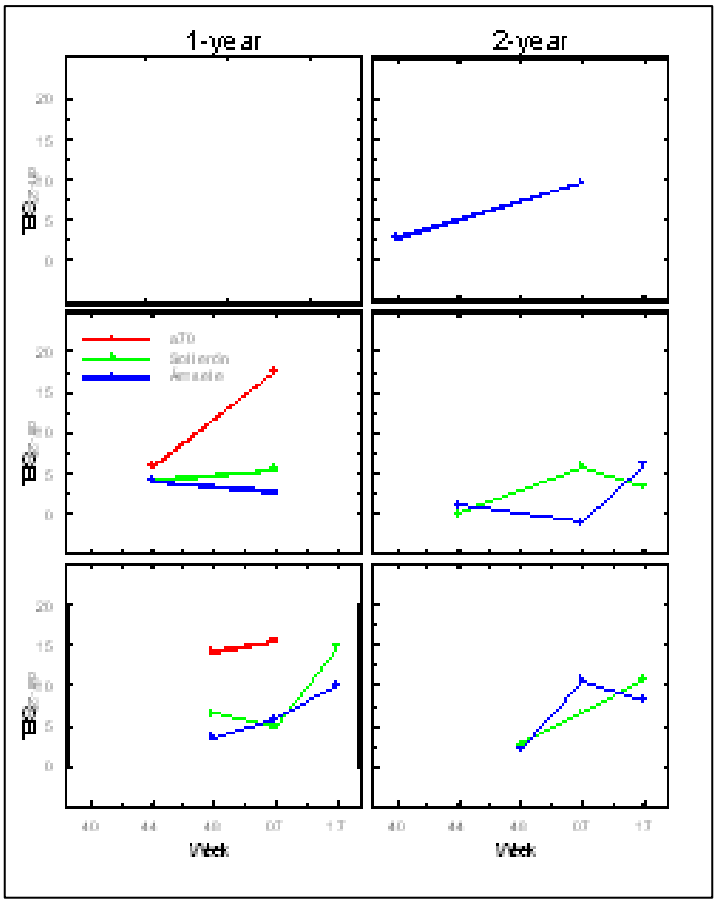


Figure 3.29. Shoot freezing tolerance, ($SEL_{diff-25}$) of 1- and 2-year-old Scots pine seedlings of three provenances (Table 2.2). The freezing tolerance was measured when seedlings were put into frozen (-5 °C) storage (week 40, 44 and 48), in the middle of the storage period (week 7) and at the end of

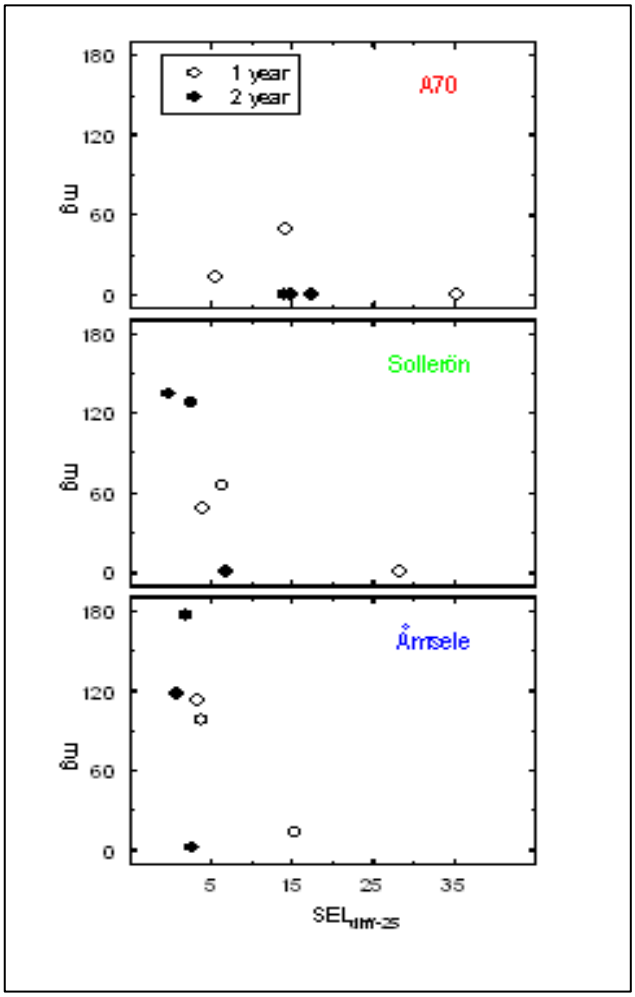


Figure 3.30. Shoot freezing tolerance ($SEL_{diff-25}$) measured on 1- and 2-year-old Scots pine seedlings of three provenances (Table 2.2) when they were put into frozen (-5 °C) storage versus root growth capacity, (mg/5 seedlings) measured after storage. Seedlings were stored in week 40, 44 and 48, 2003 and root growth capacity was measured

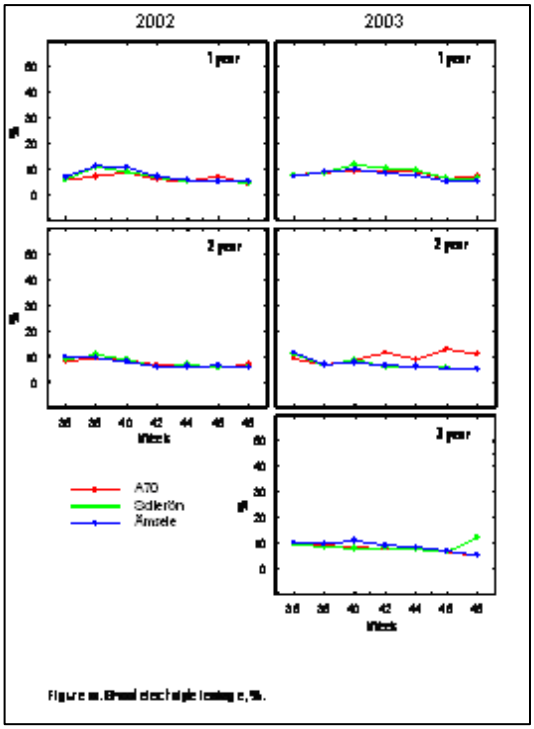


Figure 3.31 Shoot electrolyte leakage (%) of 1-, 2- and 3-year-old Scots pine seedlings of three provenances (Table 2.2) in 2002 and 2003. N = 15

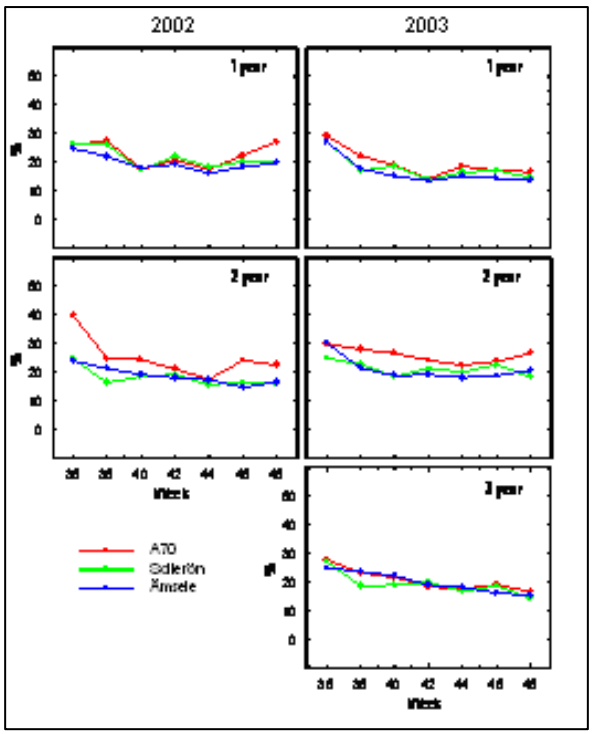


Figure 3.32. Root electrolyte leakage (%) of 1-, 2- and 3-year-old Scots pine seedlings of three provenances (Table 2.2) in 2002 and 2003. N = 5

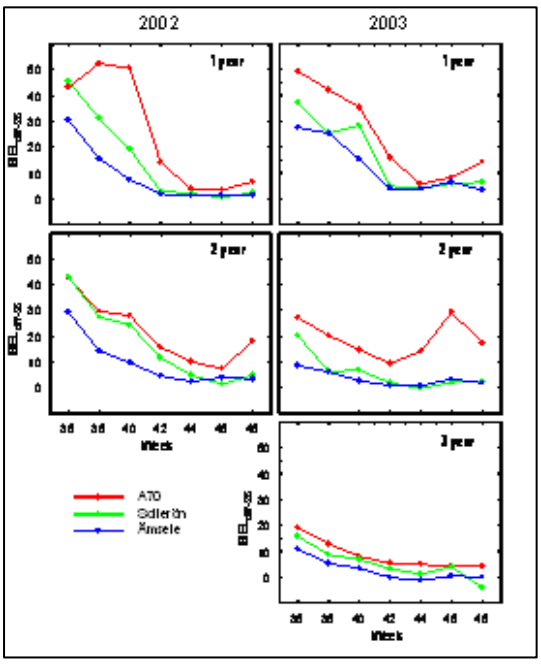


Figure 3.33. Shoot freezing tolerance, $SEL_{diff-25,}$ of 1-, 2- and 3-year-old Scots pine seedlings of three provenances in 2002 and 2003. N = 15

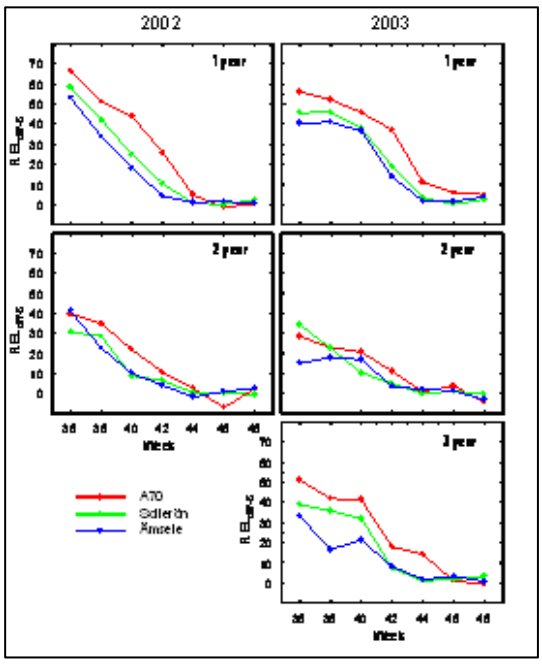


Figure 3.34. Root freezing tolerance, REL_{diff-5}, of 1-, 2- and 3-year-old Scots pine seedlings of three provenances in 2002 and 2003. N = 5

seedlings. The Swedish 1-year-old seedlings however showed a decline in the number of days to bud burst from week 36 to 44.

Table 3.2. Number of seedlings without apical bud at the start of bud break tests for 1-year-old Scots pine seedlings of three provenances (Table 2.2). N = 15

Week	A70		Sollerön		Åmsele	
	2002	2003	2002	2003	2002	2003
36	9	10	5	9	1	9
38	14	11	8	3	5	2
40	9	7	0	2	0	0
42	13	11	0	4	0	3
44	14	7	9	4	0	1
46	14	7	9	4	0	0
48	14	7	1	1	0	2

The 2- and 3-year-old seedlings of the two Swedish provenances displayed true dormancy early in autumn. The seedlings did not break bud within 90 days. In fact, some of them did not break bud even in 6 months (not shown). The decline in days to bud burst was quick between weeks 38 and 44. At week 44 the numbers of days to bud burst was approximately 25 and did not decline further. Due to lack of seedlings the A70 seedlings were only tested once as 2-year-old and not at all as 3-year-old seedlings. The 2-year-old A70 seedlings tested in 2002 showed the same pattern as the 1-year-old Swedish seedlings i.e. they did not develop any deep dormancy but showed a decline in days to bud burst from week 36 to 48.

Bud dormancy – commercial seedlings
Many of the commercially grown 1-year-old seedlings had, just as the standard seedlings, not set bud when they were taken into the greenhouse for bud break tests. The influence of photo period on bud set was apparent within these seedlings as well. There was a difference in the number of seedlings without buds between Våge and Bogrundet, provenances with similar geographical origin but grown at different locations. Våge seedlings, grown far south to their geographical origin, had set buds to a larger extent than Bogrundet seedlings in week 41 and 43 (Table 3.3).
The Våge seedlings though only 1-year-old displayed deep dormancy (Figure 3.38). Twelve of the 15 seedlings did not break bud within 90 days in growth promoting climate. The two other provenances Bogrundet and Gotthardsberg show the same pattern (Figure 3.38) as the Swedish 1-year-old standard provenances that are shown in Fig 3.36 and 3.37.

Table 3.3. Number of seedlings without apical bud at the start of bud break tests for commercially grown Scots pine seedlings of three provenances (Table 2.1). N = 15

Week	Number of seedlings		
	Gotthardsberg	Våge	Bogrundet
39		0	
41	3	1	10
43	5	1	11
45	4		9

3.7 Provenance comparison for beech
Experiments 2002-2003
The length of the seedlings was rather variable (Figure 3.39). Although the seedlings sown in 2001 were taller than those sown in 2002, the differences were quite small and the variation within a year was rather large.
The bare rooted beech seedlings all developed frost tolerance (-25°C). No differences between the provenances were found (Figure 3.40A and B).

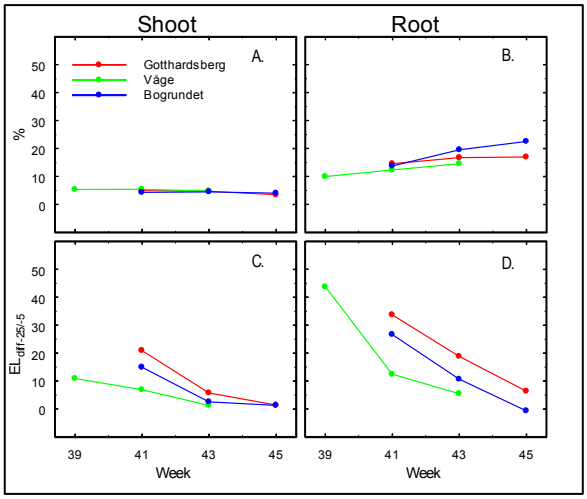


Figure 3.35. Shoot (A) and root (B) electrolyte leakage, %, and shoot, $SEL_{diff^{-}25}$, (C.) and root, $REL_{diff^{-}5}$, (D) freezing tolerance of commercially grown 1-year-old Scots pine seedlings of three provenances. Shoot N = 15; Root N = 5

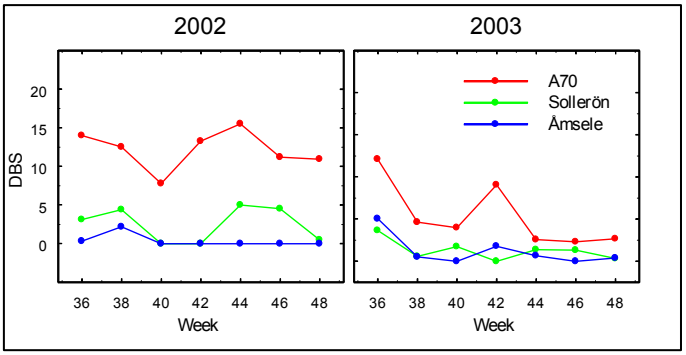


Figure 3.36. Number of days to bud set (DBS) for 1-year-old Scots pine seedlings of three provenances. Seedlings were taken from outdoor conditions and placed in growth promoting climate in a greenhouse biweekly from week 36 to week 48. N = 15

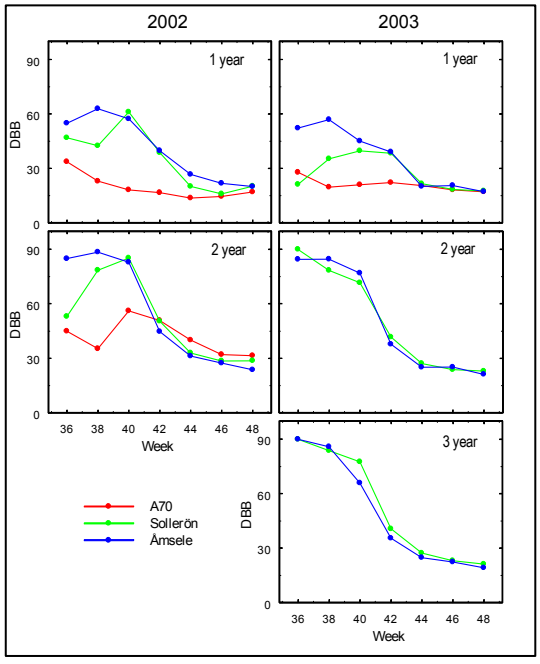


Figure 3.37. Number of days to bud burst (DBB) for 1-, 2- and 3-year-old Scots pine seedlings of three provenances. Seedlings were taken from outdoor conditions and placed in growth promoting climate in a greenhouse biweekly from week 36 to week 48. N = 15

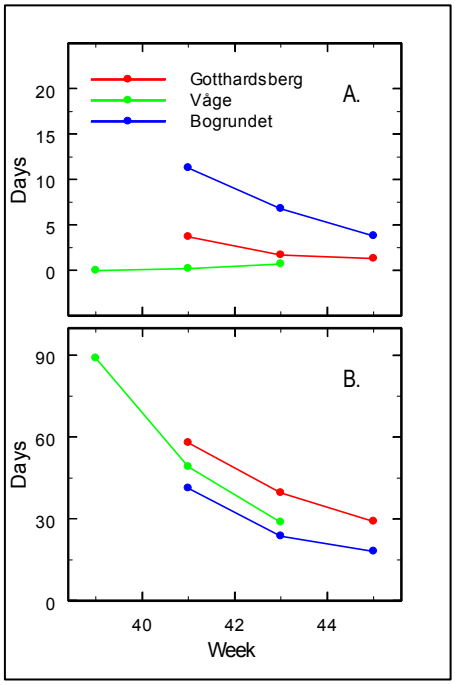


Figure 3.38. Number of days to bud set (A) and bud burst (B) for commercially grown Scots pine seedlings of three provenances (Table 2.1) when taken in from outdoor conditions and placed in growth promoting climate in a greenhouse in autumn 2003. N= 15

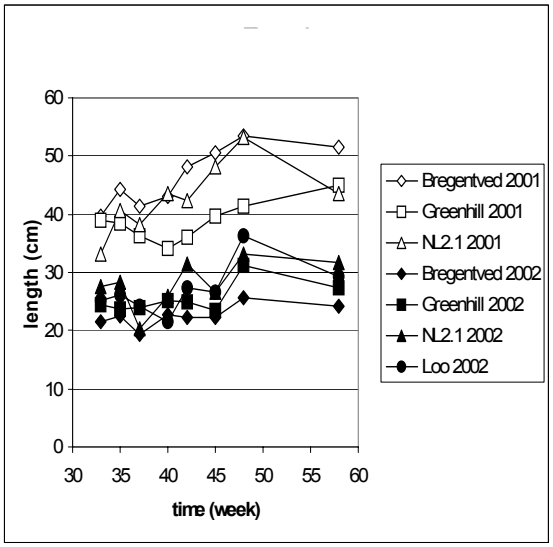


Figure 3.39. Length (cm) of the 1- and 2-year-old seedlings (sown in 2002 and 2001, respectively) of 'Bregentved', 'Greenhill', 'NL2.1' and 'Het Loo'.

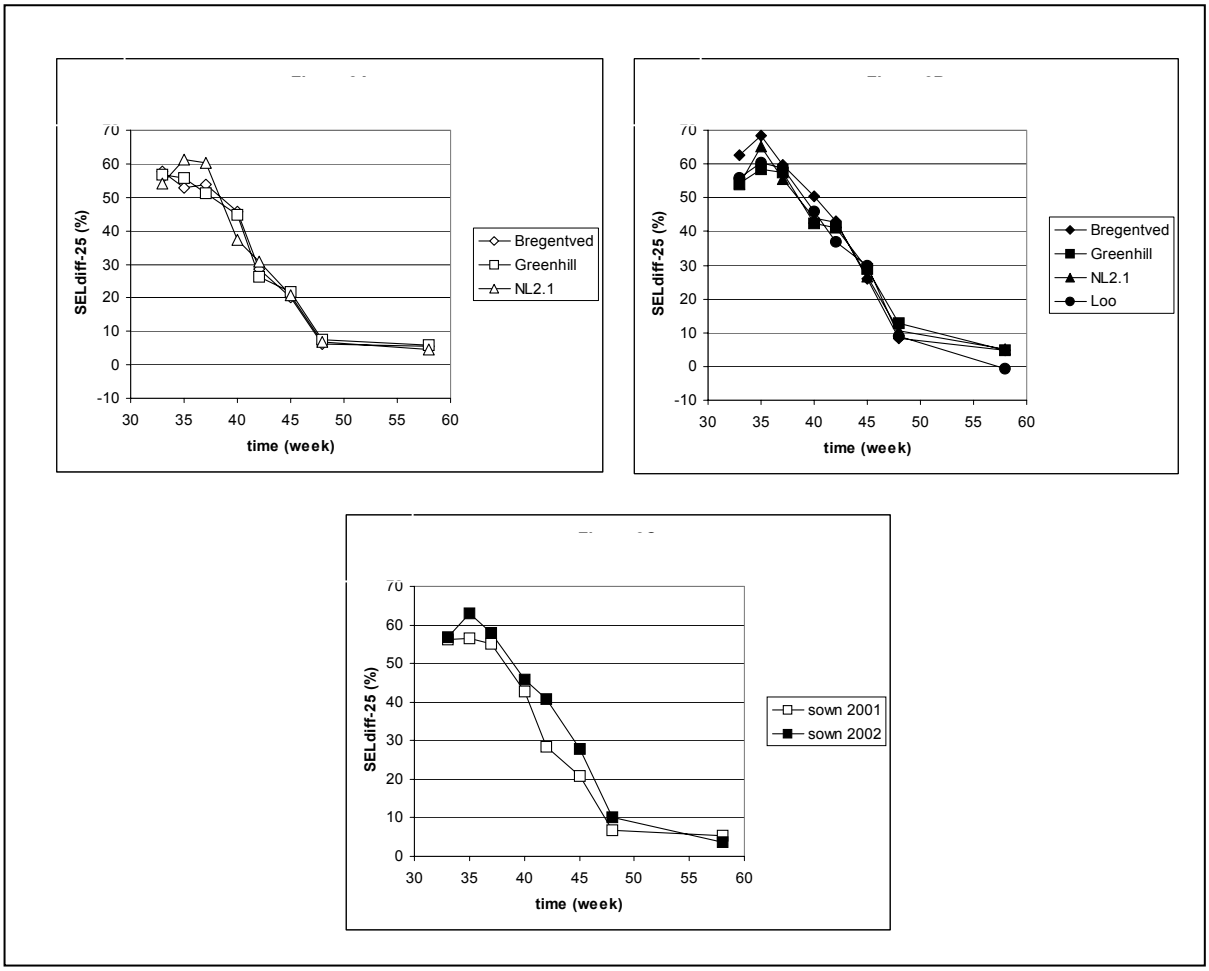


Figure 3.40.
$Sel_{diff\text{-}25}$ of 1- (B) and 2- (A) year-old seedlings of three or four provenances and of the averaged provenances (C). Seeds were sown in 2001 (A) or in 2002 (B).

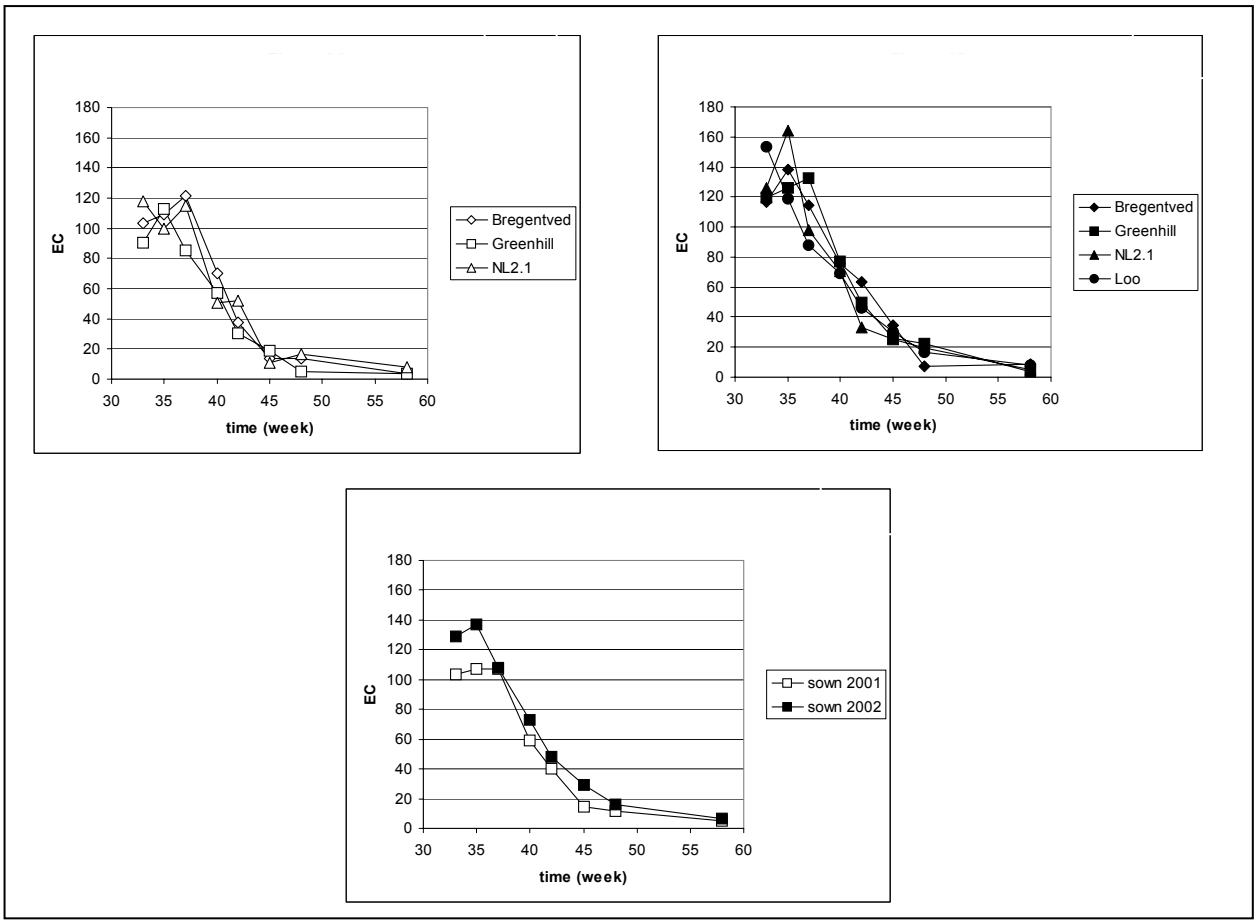


Figure 3.41.
EC of 1- (B) and 2- (A)-year-old seedlings of three or four provenances sown in 2001 (A) or in 2002 (B) and of the averaged provenances sown in 2001 (open symbols) and 2002 (closed symbols) (C).

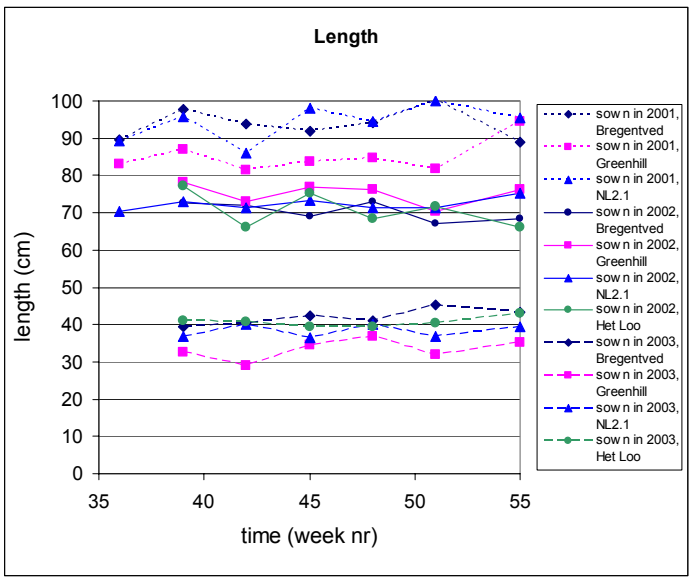


Figure 3.42. Length of 1-, 2-, and 3-year-old seedlings (sown 2003, 2002 and 2001, respectively) of the provenances 'Bregentved'.

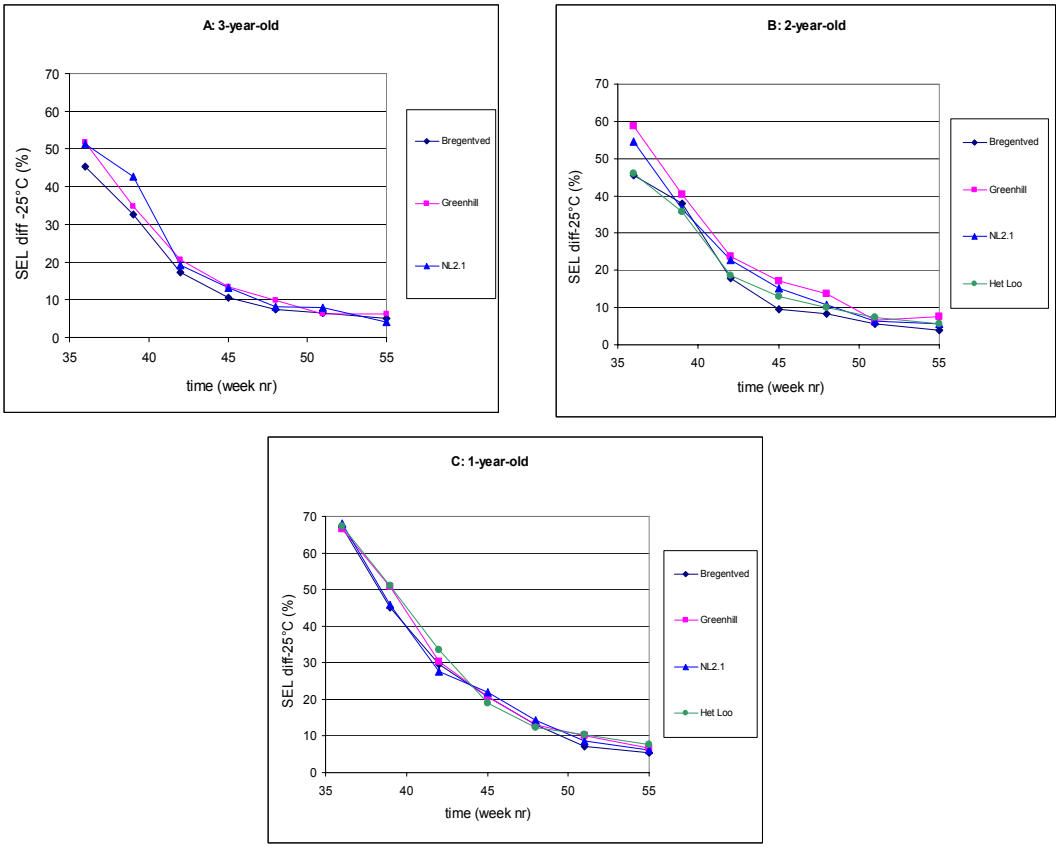


Figure 3.43.
$SEL_{diff\text{-}25°C}$ of seedlings of three or four provenances sown in 2001 (A), 2002 (B) and 2003 (C).

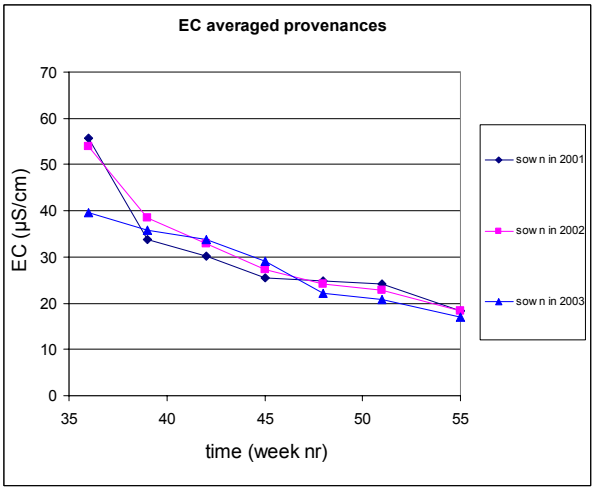


Figure 3.46. Average EC of all provenances. Seeds were sown in 2001, 2002 and 2003 (3-, 2- and 1-year old seedlings, respectively).

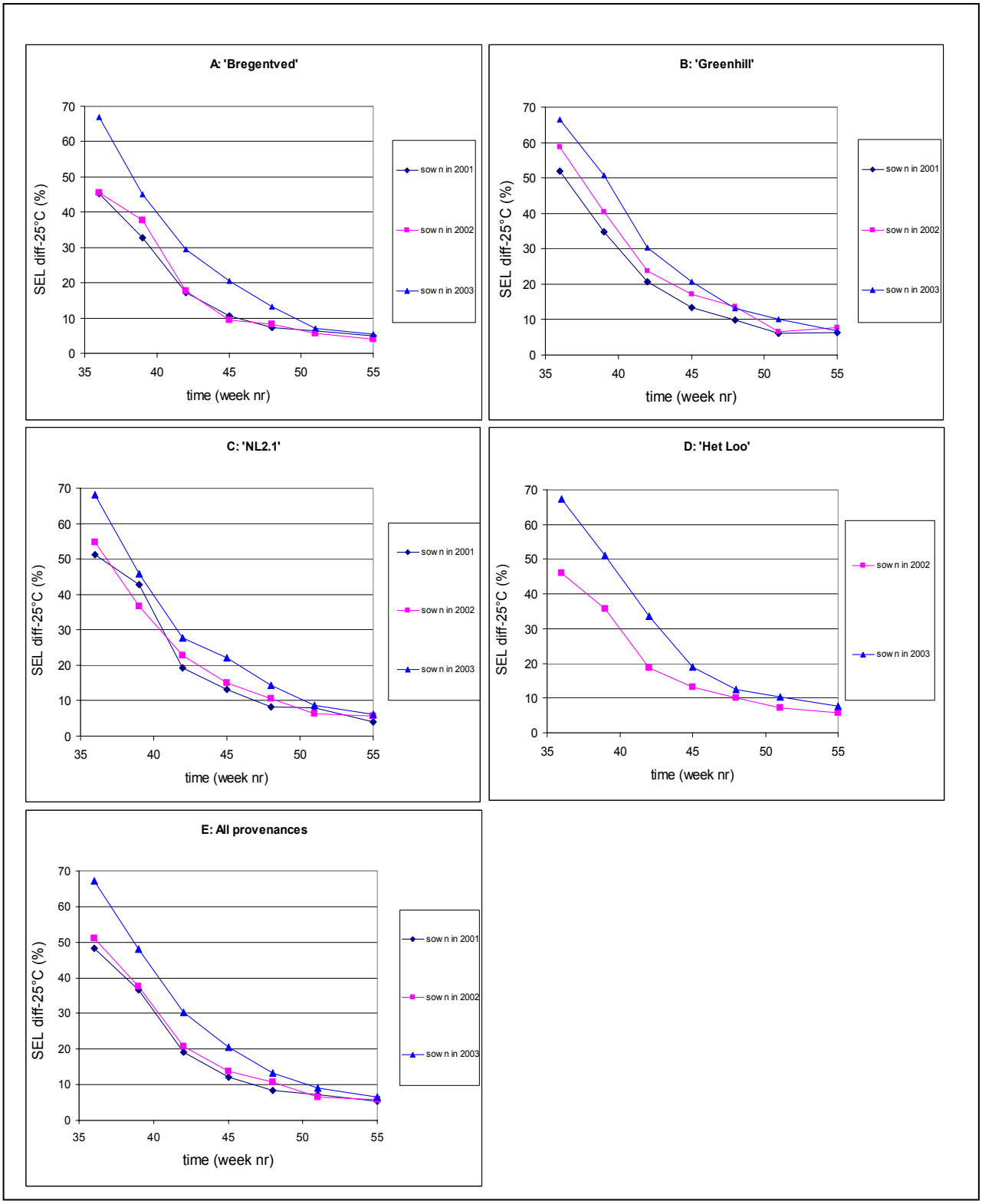


Figure 3.44.
$SEL_{diff\text{-}25°C}$ of seedlings of 'Bregentved' (A), 'Greenhill' (B), 'NL2.1' (C) and 'Het Loo' (D) sown in 2001, 2002 or 2003 and of the averaged provenances (E).

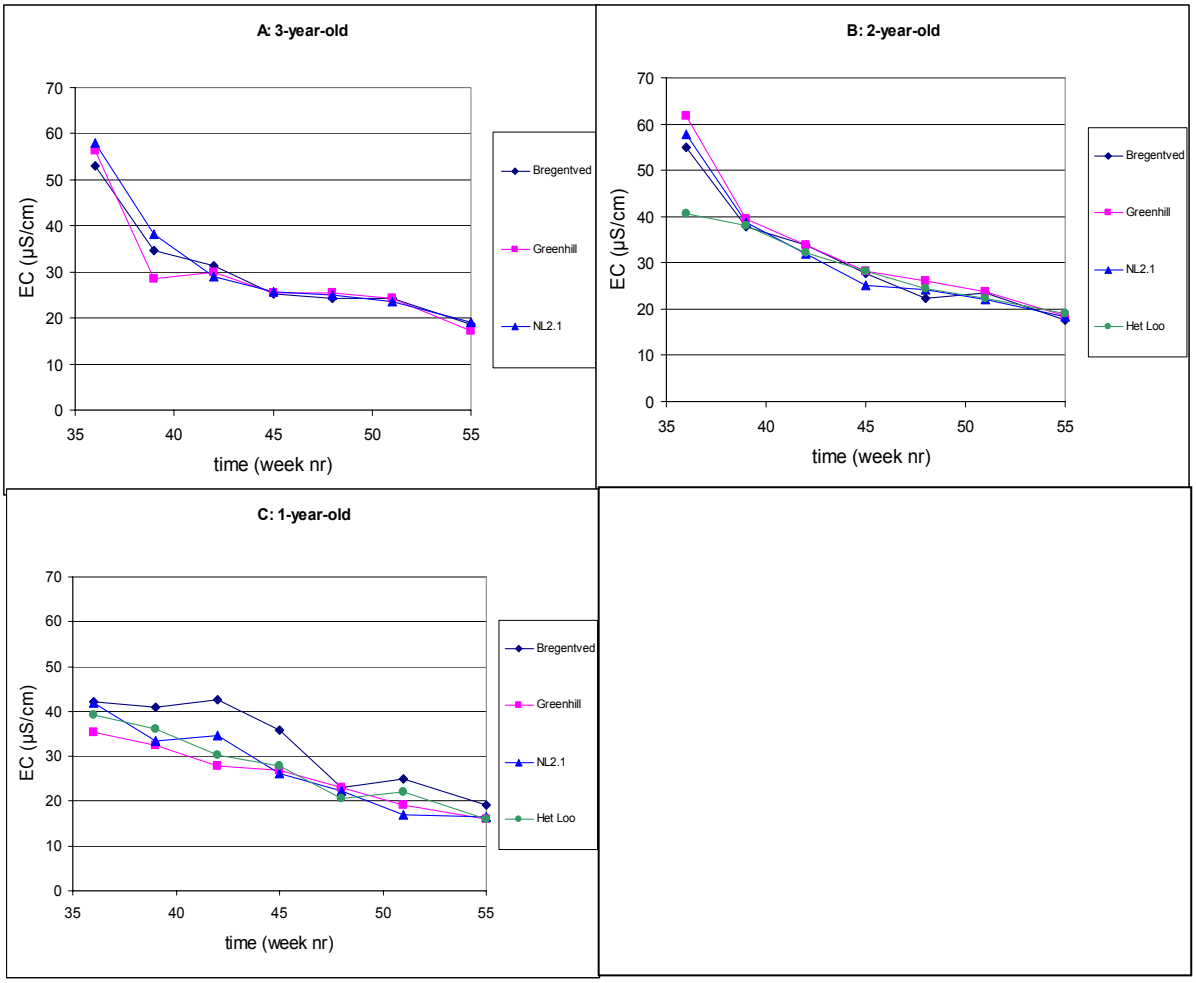


Figure 3.45.
EC of seedlings of three or four provenances sown in 2001 (A), 2002 (B) or 2004 (C).

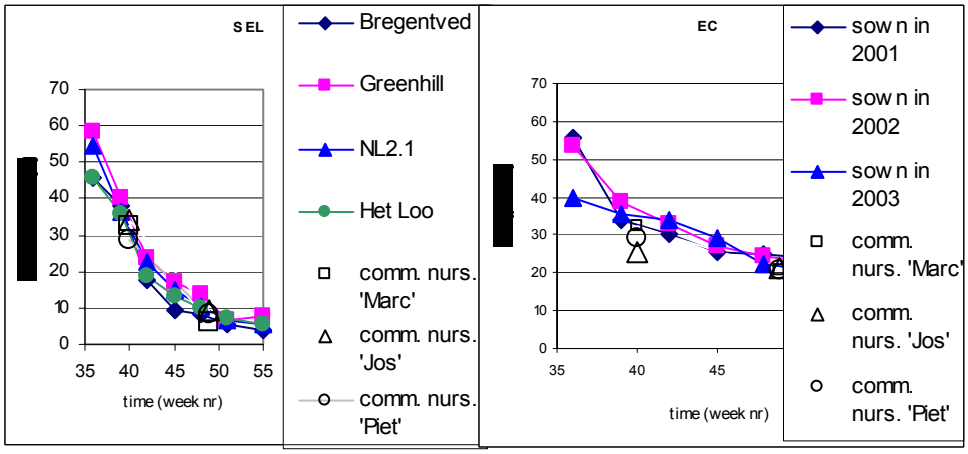


Figure 3.47. $SEL_{diff\text{-}25°C}$ (A) and EC (B) of seedlings of three commercial nurseries ('Marc', 'Jos' and 'Piet') in relation to the seedlings of the four provenances used in this study.

Seedlings germinated in 2001 (Figure 3.40A) stopped acclimatizing around the end of November (week 48), reaching the assumed threshold Seldiff -25 of about 5%.
The seedlings sown in 2002 also showed no differences in the development of frost tolerance between the provenances (Figure 3.40B), but the acclimation was slower than in seedlings one year older, and seemed to continue between week 48 2002 and week 6 2003. To visualize the difference in acclimatizing between the two sowing years Figure 3.40C shows the averaged values of all provenances.
The EC showed a tendency similar to SEL. Again, no differences between the provenances were found (Figure 3.41A and B), although the variation was slightly larger. The development of frost acclimation was different for seedlings sown in 2001 and 2002, and seemed to stop around week 45 and week 48, respectively. As with SEL the younger seedlings showed the tendency to acclimatize slower (Figure 3.41C). More data are necessary to compare EC and SEL in terms of hardiness.

3.7.2 Experiments 2003-2004
The length of the seedlings within one group was rather variable (Figure 3.42). However, the different ages of the four provenances could be clustered within three different age groups. The length of the seedlings sown in 2003 was much smaller than those sown in 2001 and 2002.
The seedlings all developed frost tolerance (-25°C). However, no differences between the provenances were detected (Figure 3.43A, B and C) within their respective age groups.
One-year-old seedlings (sown in 2003) developed frost tolerance later in the season than the two- and three-year-old seedlings (sown in 2001 and 2002) (Figure 3.44A, B, C and D). No difference between the sowing years 2001 and 2002 could be detected in the development of frost tolerance, the difference between these two age groups being at least 10 days. To visualize this effect, averages of the 4 provenances are shown in Figure 3.44E.
With respect to differences in provenances the EC showed a similar tendency as SEL, viz. no difference in provenances observed (Figure 3.45A, B and C). In contrast to the experiments in the previous year the EC showed no differences in age either (Figure 3.46). This is due to a different method that was used to measure and calculate EC.

3.7.3 Samples from commercial nurseries
The results obtained with samples taken from the commercial nurseries agreed well with those of the samples taken in Noordbroek, both for SEL (Figure 3.47A) as EC (Figure 3.47B).

3.8 Transcriptional profiling of pine
Global gene expression and physiology
In order to compare gene expression to physiological characteristics, seedlings from five different provenances were planted at three different locations, and samples were taken every two weeks from week 36 to week 04. Shoot electrolyte leakage (SEL diff-25°C, SEL diff-15°C), and root electrolyte leakage (REL diff-5°C), were measured to estimate cold tolerance. Additionally, ambient temperature and day length were measured. In order to assess the effect of climatic conditions on physiology and gene expression, an experiment was performed in which seedlings were placed in climate rooms under artificial conditions.
Samples from different experiments, locations, provenances and ages were selected for gene expression analysis using a cDNA microarray. This cDNA microarray was constructed using cDNA clones from three different *Pinus sylvestris* bud cDNA libraries. One library was prepared from mRNA from buds that were harvested in December, and two libraries were prepared using subtraction, in order to enrich for cDNAs that were up-regulated in cold-treated or in quiescent buds. In total about 1900 ESTs were sequenced from the three libraries. Contig analysis revealed that certain genes were represented by a large number of cDNA clones. For example, more than 80 dehydrin cDNAs were found, that could be divided into 5 different contigs representing different genes, each containing between 12 and 27 cDNAs. Three different contigs encoding pathogenesis related proteins were found, each containing 14-17 cDNAs, while a group

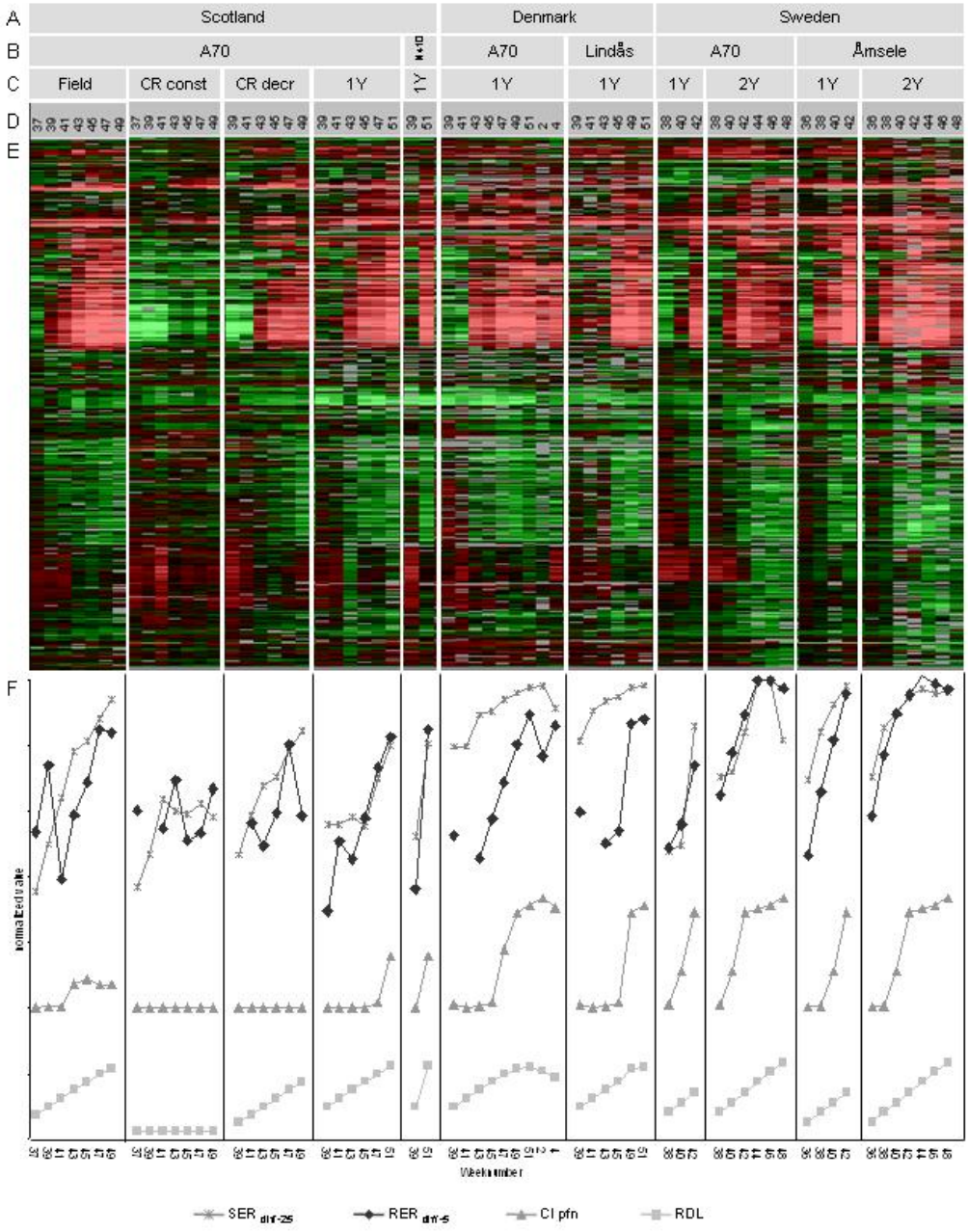


Figure 3.48. Overview of gene expression patterns and physiological characteristics of all samples analysed using the cDNA microarray. A) Location of the experiment, B) provenance used, C) field or climate room experiment (1 year old seedlings), or seedling age (field experiment). D) Sample week number. E) Gene expression in all samples (relative to a common reference made from a mixture of dormant and non-dormant buds) is shown after hierarchical clustering of the expression patterns. Red is high relative to the common reference, while green is low relative to the common reference. Shifts from green to red indicate increasing RNA levels, while changes from red to green indicate declining RNA levels. F). Physiological characteristics of the samples analysed using the microarray. The transformed physiological characteristics are shown. Asterisks: $SER_{diff\ -25}$= (100 - $SEL_{diff\ -25}$)/10, Diamonds: $RER_{diff\ -5}$ = (100 – REL $RER_{diff\ -5}$)/10, triangles: CI pfn = (hours below 4°C previous 2 weeks)/100, squares: RDL = day length /-4.

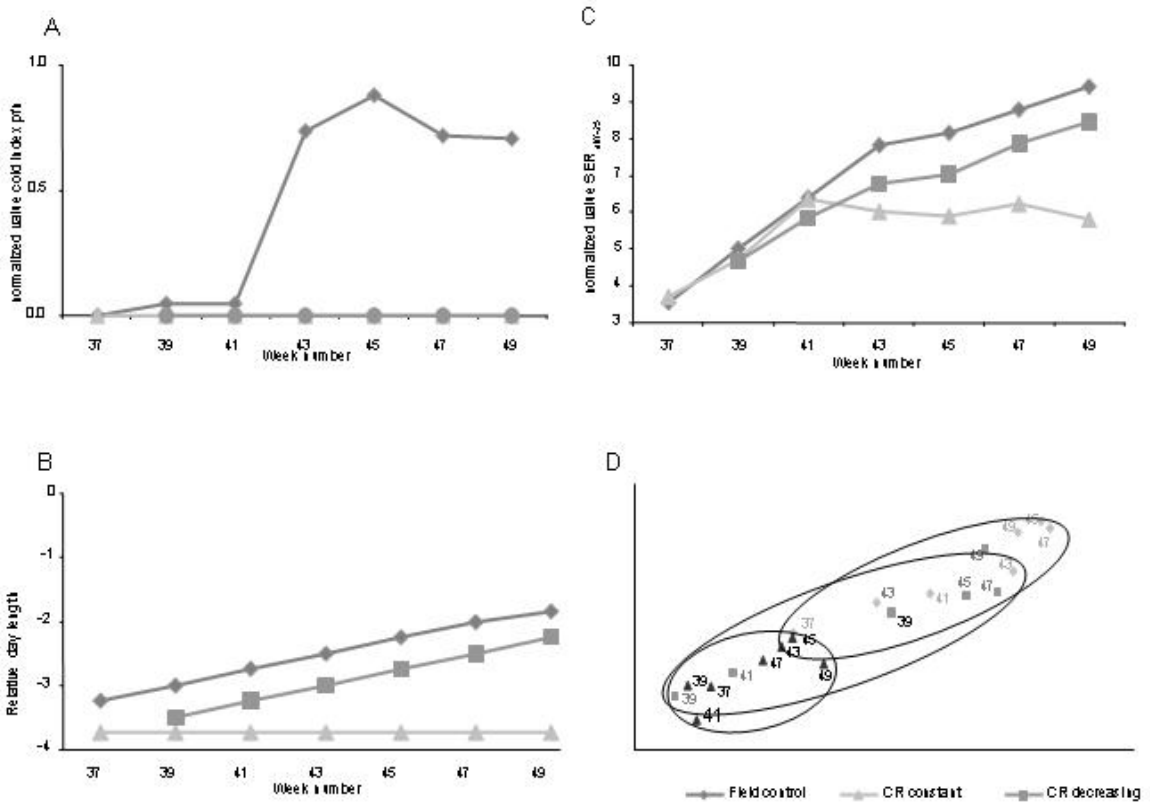


Figure 3.49. Overview of climate conditions, frost hardiness and gene expression programs in the climate room samples. Diamonds: field samples used as control; triangles: climate room with constant temperature and day length; squares: climate room with decreasing temperature and day length. A) Cold Index pfn: CI pfn = (hours below 4°C previous 2 weeks)/100; B) Relative Day length: RDL = day length /-4; C) shoot electrolyte retention: SER $_{diff\ -25}$= (100 - SEL $_{diff\ -25}$)/10; D) Principal component analysis. Week numbers as well as the duplicate values of the samples are indicated. Principal Component 1 (PC1; X-axis) explains 53.5% and PC2 (Y-axis) explains 28.1% of the observed

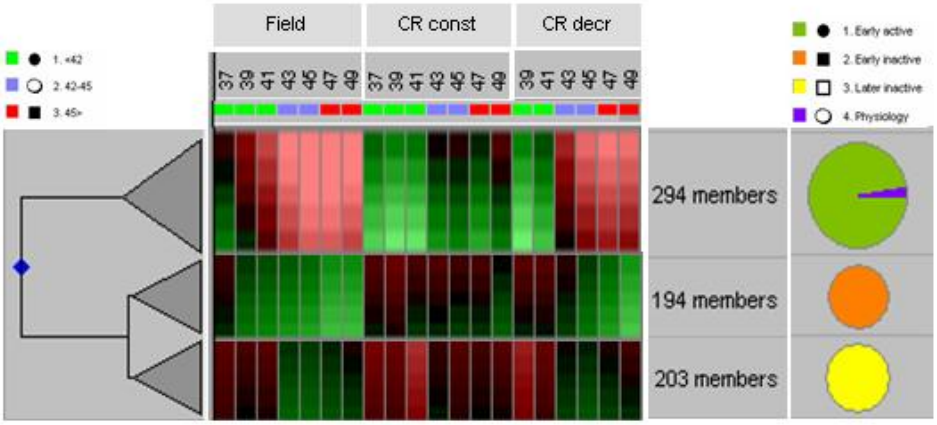


Fig 3.50 : Overview of the three expression groups using abridged Pearson clusters. High RNA levels in the various samples (relative to a common reference made from a mixture of dormant and non-dormant buds) are indicated in red, while green indicates low relative RNA levels in a sample relative to the common reference. Shifts from green to red indicate increasing RNA levels, while changes from red to green indicate declining RNA levels. Samples (week numbers) are marked with coloured rectangles indicating seasonal progression (green is early, blue is intermediate and red is late in the season). Genes are divided into three main expression groups. Group 1 (green circle) is induced in the fall, group 2 (orange circle) is inactivated early in the fall, while group 3 (yellow circle) is inactivated later in the fall. Normalized physiological parameters are indicated in blue, and cluster together with group 1 genes (blue wedge in green circle).

of 24 cDNAs with homology to a drought stress induced gene from loblolly pine (LP3: Padmanabhan et al., 1997) could be divided into 4 groups, representing putative Scots pine LP3 family members. The fact that these genes occurred so frequently in these libraries indicates that their expression level in the tissues from which the libraries were made was relatively high, suggesting that they may be involved in cold acclimation related processes. From the 1900 ESTs, 1451 were spotted on the array, together with previously characterized cDNAs, AFLP fragments and positive and negative control clones.

Figure 3.48 shows the overall results for all samples analysed. Samples from three locations (Scotland, Denmark, Sweden), four provenances (A70, N410, Lindås, Åmsele), two types of experiments (field or climate room), and two ages (one year old or two year old seedlings) were used for microarray hybridisations. The samples from each experiment are ordered chronologically by week number to visualize the changes in physiological behaviour and gene expression over time. The climate room, location, and provenance experiments are individually discussed below. In order to more easily compare physiological data to gene expression data, SEL diff-25°C was transformed to SER diff -25 (shoot electrolyte retention=100-SEL diff-25°C) and divided by 10, cold index previous fortnight (CI pfn) was divided by 100, and day length was divided by -4 to obtain relative day length (RDL). This transformation brought physiological and environmental parameters in the same numerical range as the 2log ratios for relative gene expression, while their values increased over time. Throughout this paper, we will refer to these normalized physiological parameters in comparison to gene expression data.

As can be seen in Figure 3.48E, genes can be divided into a small number of distinct groups, based on their expression patterns. A large group of genes increases in expression over time during each experiment, while another group of genes shows reduced RNA levels over time. In all field grown samples climate and physiological parameters also increase over time, with differences in the rate and onset of the increase depending on the location and provenance Late in the season the SER diff -25 and RER diff -5 are high, indicating high frost tolerance, while RDL and CI pfn are at their maximum values (reflecting shortest day length and lowest temperatures of the period measured). The increase in cold tolerance during the fall is accompanied by a major switch in gene expression, resulting in the activation of one group, and the shut-down of another group of genes. From these groups of genes a subset can be selected that can be used to molecularly characterize the seedlings.

3.8.2 Climate room samples

In order to assess the effect of temperature and day length on the acquisition of frost hardiness, seedlings grown under climate room regimes with constant (CR constant) and declining (CR decreasing) temperature and daylength were used. Identical seedlings that were grown in the field under normal fall conditions served as a control. Figure 3.49 shows the environmental conditions and the effect of the different regimes on frost hardiness. Only the seedlings that were placed outside were exposed to temperatures below 4°C (Fig 3.49A). The day length decreased both in the field and in climate decreasing (Fig 3.49B). Frost hardiness, as measured by SER diff -25 (shoot electrolyte retention) increased in all three treatments during the first 4 weeks (Fig 3.49C). From week 41 onwards, seedlings exposed to the CR constant conditions did not further develop frost hardiness, whereas the control seedlings from the field reached normal levels of frost hardiness. Seedlings that were placed in artificial fall conditions (CR decreasing) developed intermediate levels of frost hardiness (Fig 3.49C). Pine seedlings from both climate room regimes did not develop significant root frost tolerance, assessed at -5 °C, in year 2 (data not shown), indicating that plants did not develop root hardiness. These results indicate the importance of temperature and day length in the control of frost hardiness development. Interestingly, when cDNA microarray hybridisation patterns were used to characterize the samples, similar differences among the three treatments could be observed. Figure 3.49D shows a principal component analysis of the samples from the climate room experiment, based on the hybridisation patterns of all detectable spots on the array. The first and the second principle component together (explaining 81.6% of the variance among the samples), are well correlated to the level of shoot cold tolerance achieved in the

experiment (Fig. 3.49D). The samples from the CR constant are predominantly located on the lower left side of the figure, together with early samples from the CR decreasing and field experiments. The samples from the CR constant do not develop frost tolerance while the early samples from the field and CR decreasing experiments have not yet become cold tolerant. Their clustering in the PCA plot illustrates that the gene expression profiles in these samples are similar, in parallel to their comparable physiological characteristics. In contrast, the later field samples are located much more to the top right in the PCA plot, indicating their relatively high levels of frost hardiness, while later samples from the CR decreasing experiment are located in the middle section of the PCA plot, corresponding to their intermediate level of frost tolerance.

3.8.3 Selection of a descriptive gene set

The distinct differences in physiology, climatic conditions and gene expression profiles among the three treatments from the climate room experiment were exploited for the identification of a descriptive gene set that can be used to molecularly characterize samples from other experiments and locations. Using Pearson clustering, three different gene groups were identified that correlated to physiological parameters (Fig 3.50). Genes from each group display a characteristic gene expression profile during the acquisition of frost hardiness, in samples from different locations and provenances. Genes from group 1 (indicated by a green circle in Fig 3.50) increase in expression during the acquisition of frost tolerance, indicating a potential role of these genes in the hardening process. In contrast, genes from group 2 (orange circle) and group 3 (yellow circle) decreased in expression during the acquisition of frost hardiness. Genes from group 2 switched off as early as week 39 of the field samples, correlating their expression to active growth in the summer. Interestingly, these genes remained active in the CR constant experiment, and switched off much later in the CR declining experiments. Genes from group 3 were active longer into the fall season than group 2 genes, and might play a role in initiating the response to short day length and decreasing temperatures. These genes also remained active in the CR constant experiment, while their expression in the CR declining experiment was similar to that in the field.

Based on their expression pattern, these groups of genes can be linked to physiological events that occur during acquisition of frost hardiness. cDNAs from groups 2 and 3, which RNA levels decline during hardening, represent genes that are involved in active growth, and which are shut down during the quiescent winter period. Many cDNAs from these groups encode genes that are involved in translation, primary and secondary metabolism, and cell wall biosynthesis. A relatively small number of stress related genes fall within this group. Genes from group 1 however, increase in expression in parallel with the increase in cold tolerance, implicating a role in the cold response, adaptation and hardening process. This group contains a remarkably high number of stress related genes (induced by various stresses), as well as genes that are involved in biosynthesis of cellular components that can protect against stress (osmotics, antimicrobial or antifungal compounds, LEA proteins, etc). Additionally a number of primary and secondary metabolic genes, as well as transporter proteins are induced. Figure 3.51 shows an overview of the physiological and molecular processes and corresponding gene expression programs that take place during the transition from active growth to quiescence and frost hardiness.

The expression profile of the descriptive gene set provides a useful molecular signature of the various bud samples, and was therefore used to molecularly characterise the samples from the field experiments described in this paper. Additionally, individual candidate marker genes that might be useful for application in a nursery setting were selected from the descriptive gene set in the following way. Genes that displayed a high relative change in expression over the season, and whose expression showed a strong correlation to physiological parameters were chosen. For those genes that were represented by multiple spots, the average ratios of the different spots were used, making sure that spot to spot variation was low.
The genes that were selected in this manner are candidates for development into molecular markers for frost hardiness and are shown in Table 3.4. Those genes with

Table 3.4: Description of candidate marker genes for cold hardiness. Genes were selected for either a high positive or high negative overall correlation to $SER_{diff-25}$, and are and ranked according to their average Pearson correlation to $SER_{diff-25}$. For each gene the most likely gene function based on blast analysis, the functional category, the descriptive gene set expression group (see fig 3.50), the number of cDNAs on the array representing the gene, the most likely Arabidopsis homolog, the E-value from the blast on the Arabidopsis genome, and the average Pearson correlation from the three experiments described in this article are shown.

putative function (blast output)	functional category	group	nr cDNAs	At number	E-value (BLAST)	Pearson
no blast match, contig 52	unknown	1	3	NA		0,79
purple acid phosphatase	metabolism	1	2	At3g20500.1	2,00E-28	0,75
metallothionein	development/stress	1	1	At2g23240.1	7,00E-15	0,73
transcription factor E2F	transcription	1	1	At5g22220.2	6,00E-47	0,73
P. Taeda EST set C_CF388200	unknown	1	4	NA		0,73
intracellular PR protein	biotic stress	1	2	NA		0,72
no blast match, contig 50	unknown	1	5	NA		0,72
ELIP	photosynthesis	1	2	At3g22840.1	4,00E-17	0,71
ser proteinase inhibitor	protein degradation	1	3	At1g72060.1	4,00E-07	0,71
LEA protein	development/stress	1	3	NA		0,70
epoxide hydrolase	metabolism	1	1	At4g02340.1	1,00E-22	0,70
CAAT binding factor subunit	transcription	1	1	At4g14540.1	5,00E-33	0,69
defensin (gamma thionin family)	biotic stress	1	3	At2g02120.1	2,00E-09	0,69
dehydrin dhn5	abiotic stress	1	15	At1g76180.1	1,00E-07	0,68
dehydrin like	abiotic stress	1	2	At1g20440.1	1,00E-04	0,68
dehydrin dhn3 or dhn 7	abiotic stress	1	12	At5g66400.1	3,00E-07	0,68
LP-3 like A	abiotic stress	1	7	NA		0,68
aluminum induced protein	unknown	1	3	At5g19140.1	1,00E-32	0,67
LP-3 like B	abiotic stress	1	5	NA		0,66
thaumatin-like	biotic stress	1	4	At4g11650.1	6,00E-16	0,65
LP-3 like C	abiotic stress	1	2	NA		0,64
pinosylvin synthase	biotic stress/metabol	1	3	NA		0,64
LEA protein	development/stress	1	3	At1g01470.1	1,00E-22	0,63
LP-3 like D	abiotic stress	1	2	NA		0,63
sucrose synthase	osmotics	1	3	At5g49190.1	7,00E-35	0,63
beta-1,3-glucanase	defence	1	2	At2g16230.1	7,00E-14	0,56
malate dehydrogenase	metabolism	1	3	At4g00570.1	2,00E-26	0,55
LEA protein	development/stress	1	3	NA		0,50
P. Taeda EST set C_CF472479	unknown	1	2	NA		0,48
proline rich protein	cell wall	1	6	At4g12500.1	1,00E-20	0,47
class IV chitinasse	biotic stress	1	4	At3g12500.1	3E-24	0,44
PR10 protein	biotic stress	1	4	NA		0,29
dehydrin dhn2	abiotic stress	1	2	At1g20450.1	9,00E-14	0,04
aquaporin	transport	2	2	At2g37170.1	2,00E-27	-0,48
14-3-3 protein	regulators	2	2	At2g42590.1	1,00E-92	-0,49
ubiquitin	protein degradation	2	2	At5g56150.2	3,00E-74	-0,54
60S ribosomal protein L14	translation	2	1	At2g20450.1	3,00E-46	-0,58
P. Taeda EST CF668373	unknown	2	2	NA		-0,63
P. Taeda EST BE241143	unknown	3	1	NA		-0,63
acid phosphatase	metabolism	2	1	At4g29270.1	1,00E-39	-0,64
calmodulin	signal transduction	2	7	At2g27030.3	3,00E-36	-0,65
ubiquitin/ribosomal protein 27a	translation	2	3	At2g47110.1	1,00E-66	-0,67
dehydrin 1	abiotic stress	2	1	At1g20440.1	3,00E-05	-0,67
40S ribosomal protein S5	translation	2	1	At2g37270.1	2,00E-96	-0,67
ubiquitin/ribosomal protein L40	translation	2	2	At3g52590.1	2,00E-16	-0,70
ribosomal protein S7	translation	3	1	At5g16130.1	8,00E-73	-0,70
alpha tubulin 1	structure	2	1	At1g50010.1	2,00E-53	-0,70
ribosomal S26	translation	2	3	At3g56340.1	1,00E-27	-0,72
xyloglucan endotransglycosylase	cell wall	2	1	At4g03210.1	8,00E-56	-0,73
ribosomal S11	translation	2	2	At4g30800.1	4,00E-31	-0,75
P. Taeda EST CF389725	unknown	2	1	NA		-0,75

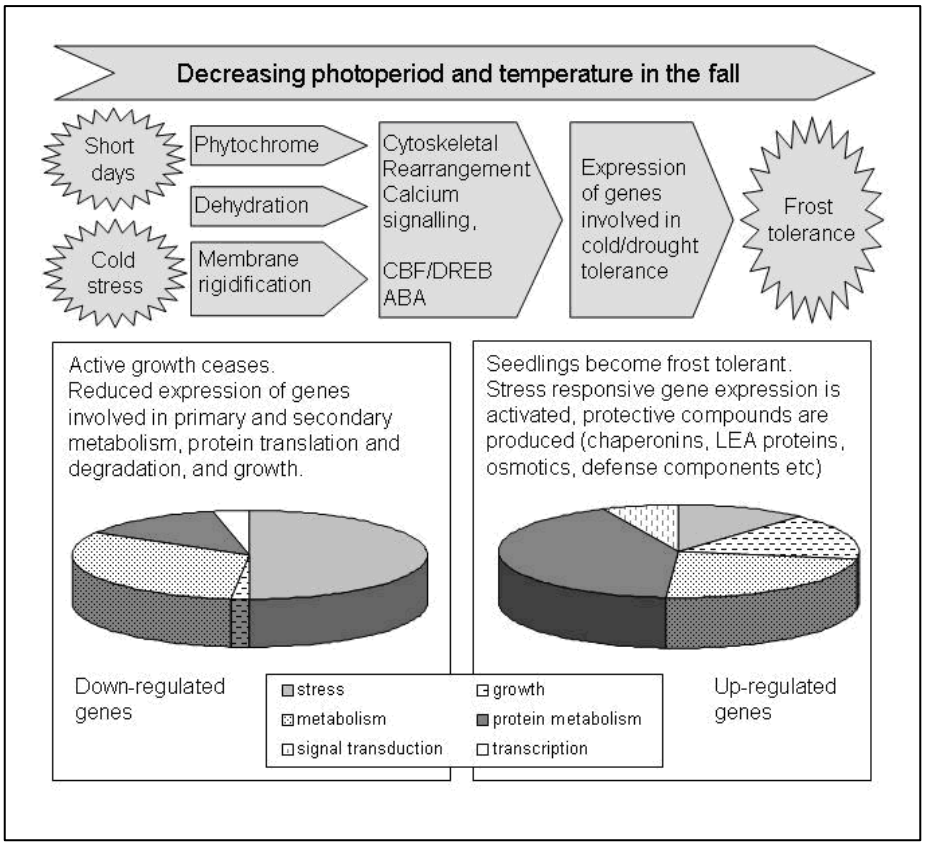


Fig 3.51: Molecular and physiological events in the transition from active growth to quiescence and frost hardiness. Shortening photoperiod and reducing temperatures trigger physiological (stress) responses leading to quiescence and frost tolerance. Lower panels show the functional classification of the most highly up-regulated (60) or down-regulated (43) genes on the microarray.

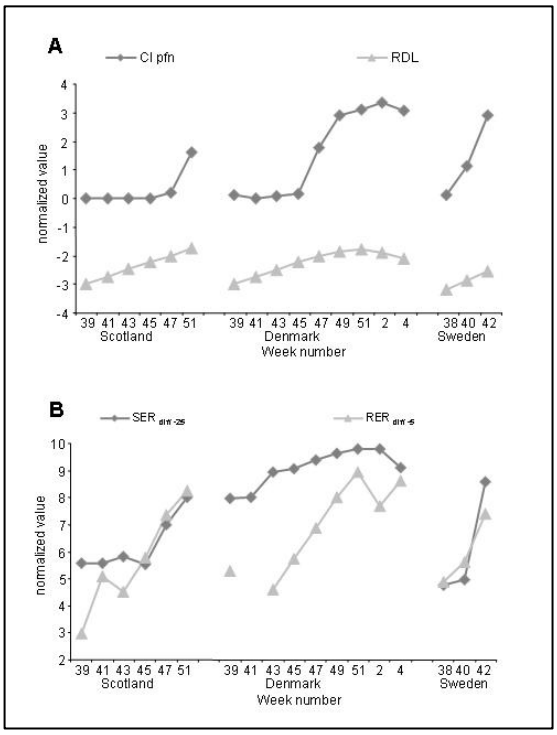


Figure 3.52 Overview of climate conditions and frost hardiness in 1 year old A70 seedlings in the same season at 3 different locations. A): climate conditions. Diamonds: Cold Index pfn: CI pfn = (hours below 4°C previous 2 weeks)/100; triangles: Relative Day length: RDL = day length /-4; B): Frost hardiness. Diamonds: shoot electrolyte retention: $SER_{diff\ -25}$= (100 - $SEL_{diff\ -25}$)/10; triangles: Root electrolyte retention: $RER_{diff\ -5}$= (100 - $REL_{diff\ -5}$)/10.

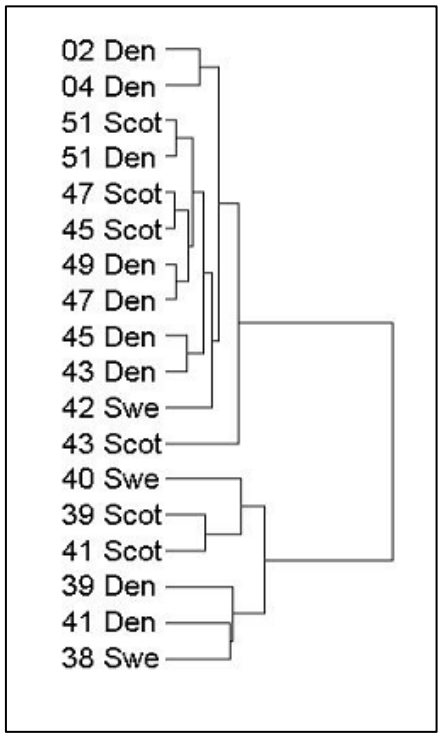


Figure 3.53 Hierarchical clustering of A70 samples from three different locations based on the expression of the descriptive gene set. Location code (Swe=Sweden, De=Denmark, Sc=Scotland) and week numbers indicate the samples.

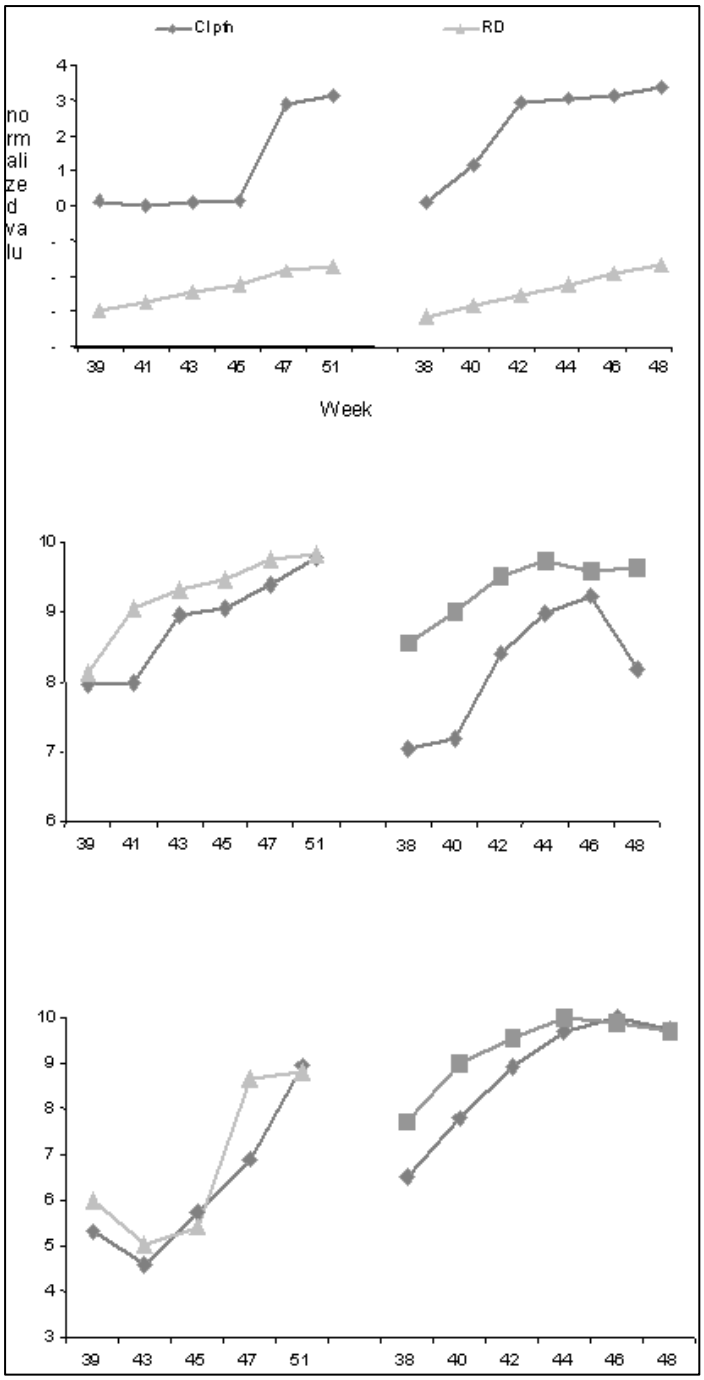


Figure 3.54. Comparative overview of climate conditions and frost hardiness of two provenances under identical conditions at two locations. A): Climate conditions in Denmark and Sweden during the experiments. Diamonds: Cold Index pfn: CI pfn = (hours below 4°C previous 2 weeks)/100; Triangles: Relative Day length: RDL = day length /-4; B): Shoot electrolyte retention ($SER_{diff\ -25}$) of the two Danish and Swedish provenances. C) Root electrolyte retention ($RER_{diff\ -5}$) of the two Danish and Swedish provenances.

the highest average positive Pearson correlation to SERdiff-25 (representing up-regulated genes) are at the top of the list, while the genes with the highest average negative Pearson correlation to SERdiff-25 (representing down-regulated genes) are at the bottom of the list. The average expression ratios of the genes in all the climate room samples and their relation to the physiological end environmental parameters in this experiment are shown in Table 3.5. At the bottom right of Table 3.5, the Pearson correlation among the various physiological and environmental parameters is shown. SER diff -25 and SER diff -15 are highly correlated, but do not correlate well with RER diff -5. This is possibly due to the fact that in the climate room regime, no root frost hardiness developed. There was a fairly good correlation between SER diff -25 and RDL however. This also explains why genes which expression profile strongly correlated to SER diff -25 also showed a good correlation to SER diff -15 and RDL.

3.8.4 Location effect:

In order to assess the predictive value of the descriptive gene set at different locations, samples from the standard provenance A70 were grown and analyzed simultaneously in Scotland and Denmark, and compared to an experiment performed the following year in Sweden. As can be seen in Figure 3.52, the environmental conditions at these three locations were quite different. Although Denmark and Scotland are roughly on the same latitude, and have very similar day lengths, their temperatures differ. In Sweden, day length declines more rapidly, while temperature drops much earlier in the season. (Fig 3.52A). These conditions affect the physiological behaviour of the seedlings (grown from the same seed lot under similar greenhouse conditions), as can be seen in Figure 3.52B. In Scotland the native A70 seedlings take much longer to develop cold tolerance, while in Sweden the increase in cold tolerance is much earlier and more rapid (Fig 3.52B).

When the samples from the location experiment are clustered based on the expression profiles of the descriptive gene set, it can be seen that they are distributed into two main groups, a late and an early group respectively. The cluster on the top of Figure 3.53 contains the samples from week 42 onwards, which are more cold tolerant samples, while the cluster on the bottom contains samples from week 38 through 41, which are not yet cold tolerant.

Interestingly, it seems that the overall expression of the descriptive gene set in individual samples is more highly influenced by sampling date (reflecting local decrease in day length and temperature) than by experiment location. In the physiological data the “location effect” is more profound, possibly also because physiological tests were done independently at each location using the same protocol, while microarray experiments were all performed in the same lab at PRI.

In the climate room experiment clear differences were found both in physiological and in gene expression characteristics, but at the three different field locations from this experiment, these differences were relatively small. Since the samples in Sweden were sampled in different weeks than those in Scotland and Denmark, it is possible that the seedlings in Sweden were slightly ahead of the others in their gene expression program. Even though the overall effect on gene expression of the descriptive gene set is mainly influenced by sampling date, the expression of the individual candidate marker genes shows a good correlation to the physiological parameters (Table 3.6), indicating their potential usefulness as molecular markers for frost hardiness. In this experiment, RER diff -5 showed a relatively good correlation to both SER diff -25 and RDL, indicating that root hardening progressed at a similar rate to shoot hardening, unlike in the climate room experiment. Interestingly, in this experiment gene expression showed a better correlation with RER diff -5 than with SER diff -25 (Table 3.6).

3.8.5 Provenance effect

In order to assess the predictive value of the descriptive gene set for different provenances under the same climatic conditions, samples from the standard provenance A70 and a local provenance were compared in a single experiment. In Denmark, A70 was compared to the local provenance Lindås, while in Sweden, A70 was compared to the local provenance Åmsele. As can be seen in Figure 3.54A, the temperature in Sweden starts declining much earlier than in Denmark (earlier increase

Table 3.5 Expression of candidate marker genes in the climate room experiment and their correlation to physiological and environmental characteristics. Columns contain: gene name, sample week number in the various experiments, and Pearson correlations of the expression profile to the corresponding physiological and environmental parameters of the samples

Table 2	field control, second year, scotland, A70							climate room, constant conditions							climate room, decreasing day length and temp						Pearson correlation to physiology					
week number	37	39	41	43	45	47	49	37	39	41	43	45	47	49	39	41	43	45	47	49	SER-25	RER-5	SER-15	CI	CI pfn	RDL
no blast match, contig 52	-0,29	0,75	2,46	2,12	3,16	3,16	2,48	-0,81	-0,41	-0,28	-0,29	-0,25	-0,40	0,14	-0,26	-0,86	0,38	2,22	2,36	3,40	0,82	0,38	0,87	0,57	0,62	0,92
purple acid phosphatase	-0,90	-0,72	0,58	2,00	1,83	2,08	1,59	-1,63	-1,21	-1,26	-1,05	-0,81	-0,97	-0,95	-1,10	-0,78	0,37	1,22	1,29	0,84	0,86	0,39	0,88	0,66	0,74	0,93
metallothionein	-1,22	0,74	0,96	2,31	2,09	3,27	2,70	-2,08	-1,47	*	-0,26	0,15	-0,57	-0,48	-1,46	-1,64	0,07	1,43	1,98	3,11	0,90	0,47	0,92	0,62	0,64	0,89
transcription factor E2F	-0,53	0,95	*	2,40	3,29	2,83	2,47	-0,58	0,04	-0,37	0,39	0,54	0,20	0,30	-1,27	-0,74	1,56	2,54	2,15	2,06	0,85	0,51	0,88	0,61	0,69	0,85
P. Taeda EST set C_CF388200	-1,06	-0,81	-0,28	1,43	2,40	2,89	2,42	-1,33	-1,23	-0,87	-0,91	-1,24	-1,34	-1,36	-1,43	-1,02	-1,76	-0,44	0,69	2,58	0,83	0,55	0,87	0,76	0,79	0,89
intracellular PR protein	-0,56	-0,28	-0,14	1,31	2,33	2,30	1,93	-1,90	-1,88	-1,11	-1,83	-1,44	-1,88	-1,84	-1,64	-1,76	-0,62	-0,17	0,78	1,64	0,81	0,49	0,84	0,73	0,78	0,96
no blast match, contig 50	-1,20	-0,83	-1,01	0,06	1,21	2,85	1,94	-1,52	-1,45	-1,04	-1,14	-1,00	-1,40	-1,31	-1,29	-1,24	-1,29	-1,22	-0,42	2,74	0,79	0,54	0,81	0,75	0,69	0,82
ELIP	0,25	0,42	2,24	3,76	3,29	4,63	4,57	0,37	0,75	0,73	-0,11	0,22	0,30	0,12	0,74	-0,11	0,39	1,75	2,51	3,11	0,83	0,48	0,89	0,80	0,81	0,91
ser proteinase inhibitor	0,03	0,38	-0,64	0,53	2,67	2,38	2,47	-0,76	-1,14	-0,45	-1,00	-0,65	-1,46	-0,82	-0,56	-1,09	-0,86	-0,58	1,08	1,50	0,71	0,67	0,75	0,80	0,78	0,87
LEA protein	-1,82	-1,06	-0,13	0,60	1,47	1,99	1,60	-2,15	-2,46	-2,92	-1,17	-0,18	-1,18	-0,95	-2,19	-2,44	-1,65	-0,56	-0,26	1,53	0,82	0,47	0,84	0,71	0,72	0,83
epoxide hydrolase	-1,70	-0,18	0,82	1,89	2,75	2,51	*	-2,23	*	-2,21	-1,88	-1,20	-2,23	-0,76	-2,58	-2,18	0,17	1,40	1,99	1,82	0,81	0,38	0,86	0,61	0,64	0,92
CAAT binding factor subunit	-0,17	0,94	1,52	2,33	2,98	2,73	2,59	-0,99	-1,24	-1,03	-0,39	-0,13	-0,54	0,13	-0,90	-0,39	1,10	2,16	2,06	2,30	0,83	0,43	0,85	0,62	0,68	0,94
defensin (gamma thionin family)	0,40	0,80	2,51	2,90	4,13	3,42	3,14	0,51	-0,25	0,07	0,73	1,05	0,53	1,30	0,33	0,19	0,86	2,70	2,58	3,20	0,83	0,41	0,87	0,64	0,71	0,88
dehydrin dhn5	-1,57	0,28	1,37	2,48	2,88	2,83	2,92	-2,89	-4,25	-3,78	-1,67	-1,33	-2,20	-1,04	-3,96	-2,80	0,68	1,88	2,29	2,09	0,80	0,43	0,82	0,58	0,63	0,90
dehydrin like	-1,32	0,56	1,44	2,56	3,23	3,03	2,80	-2,40	-2,87	-2,38	-0,80	-0,50	-1,19	-0,41	-2,60	-2,03	0,81	2,41	2,56	2,51	0,84	0,44	0,86	0,58	0,63	0,90
dehydrin dhn3 or dhn 7	-0,76	1,21	2,06	2,94	3,41	3,18	2,97	-2,22	-2,98	-2,35	-0,99	-0,68	-1,14	-0,29	-2,69	-2,09	1,45	2,48	2,45	2,36	0,79	0,40	0,81	0,57	0,63	0,90
LP-3 like A	0,19	0,79	1,80	2,86	3,37	3,27	2,91	-0,68	-1,07	-1,09	0,01	0,25	0,00	0,50	-0,96	-0,23	1,50	2,27	2,59	2,28	0,83	0,44	0,85	0,63	0,70	0,92
aluminum induced protein	-0,59	-0,26	-0,26	-0,09	1,18	0,77	0,37	-1,14	-0,91	-1,13	-0,07	0,16	-0,12	0,15	-1,72	-0,73	0,37	0,67	0,91	0,65	0,76	0,44	0,73	0,42	0,45	0,67
LP-3 like B	-0,17	0,96	1,79	2,56	3,53	3,18	2,94	-2,14	-2,53	-2,44	-0,10	0,08	-0,53	0,72	-2,83	-0,85	1,69	2,84	2,54	2,77	0,80	0,40	0,79	0,54	0,59	0,86
thaumatin-like	-0,47	-0,87	1,41	1,82	2,89	2,91	1,86	-2,01	-2,38	-1,92	-1,22	-0,97	-1,81	-0,43	-1,77	-1,99	-0,94	0,86	1,44	1,81	0,80	0,42	0,83	0,65	0,72	0,91
LP-3 like C	-0,47	0,96	1,62	2,57	3,67	3,18	2,90	-2,98	-2,94	-3,39	-0,13	0,08	-0,32	0,64	-3,59	-0,86	1,84	2,87	2,42	2,98	0,79	0,38	0,76	0,51	0,56	0,83
pinosylvin synthase	-0,02	-1,25	-0,32	0,66	0,56	1,77	1,64	-0,75	-1,12	-0,63	-0,99	-0,51	-0,94	-0,67	-0,68	-0,81	-0,97	-0,93	-0,24	0,88	0,72	0,47	0,74	0,83	0,78	0,80
LEA protein	0,04	0,83	0,61	2,40	1,56	1,82	1,74	-0,37	-0,52	-0,15	-0,33	-0,16	-0,34	-0,03	-0,32	0,13	1,03	1,17	1,37	1,08	0,79	0,44	0,82	0,63	0,74	0,92
LP-3 like D	-0,38	0,59	1,43	2,59	2,44	2,80	2,30	-1,23	-0,47	-0,24	-0,57	-0,40	-0,79	-0,54	-0,73	-1,00	0,43	2,07	2,51	2,78	0,84	0,42	0,89	0,57	0,64	0,93
sucrose synthase	-0,60	-0,47	-0,10	0,72	1,07	1,20	0,56	-1,00	-0,46	-0,73	-0,59	-0,33	-0,51	-0,83	-1,08	-0,93	-0,52	0,27	0,59	0,87	0,85	0,46	0,90	0,65	0,73	0,88
beta-1,3-glucanase	1,14	0,21	-0,05	1,60	2,38	2,21	1,25	-0,66	-0,83	-0,74	-0,82	-0,16	-1,01	-0,61	-0,80	-0,93	-0,07	0,31	0,05	0,37	0,57	0,42	0,60	0,74	0,85	0,81
malate dehydrogenase	0,13	0,39	1,69	2,34	2,37	2,21	1,79	-0,45	-0,76	-1,16	0,69	0,43	0,30	0,88	-1,08	-0,55	1,10	2,08	2,40	2,02	0,78	0,37	0,81	0,48	0,57	0,81
LEA protein	-0,23	1,24	1,51	1,12	2,31	2,35	2,79	-1,53	-2,04	-1,01	-1,73	-1,95	-2,21	-1,67	-1,28	-1,83	-0,48	-0,60	1,09	0,77	0,68	0,49	0,72	0,72	0,73	0,92
P. Taeda EST set C_CF472479	-0,52	-1,14	-0,95	0,01	-0,34	1,10	1,30	-1,16	-1,71	-0,86	-1,39	-1,18	-2,07	-1,97	-0,99	-1,24	-1,73	-1,34	-0,89	-0,83	0,58	0,56	0,62	0,87	0,79	0,76
proline rich protein	1,00	-0,11	1,05	2,59	2,18	2,19	1,32	-0,44	-1,00	-1,88	-0,13	-0,46	-0,29	-0,51	-0,52	-0,59	1,36	1,88	1,59	-1,51	0,47	0,29	0,50	0,53	0,66	0,67
class IV chitinasse	1,06	0,54	1,43	1,66	2,40	2,57	2,08	-1,07	-2,12	-1,64	-1,05	-0,06	-1,25	0,08	-1,75	-1,64	0,12	0,72	1,06	0,76	0,65	0,41	0,67	0,66	0,70	0,85
PR10 protein	2,75	2,81	4,48	4,28	4,74	4,12	4,25	0,81	-0,25	-0,35	2,15	2,48	2,09	2,83	-1,75	0,33	2,81	3,89	3,98	2,02	0,60	0,32	0,61	0,49	0,56	0,68
dehydrin dhn2	0,46	1,13	0,69	1,77	0,98	0,70	0,88	-0,68	-1,96	-2,30	-0,48	-0,80	-0,28	-0,32	-1,14	-0,14	1,48	1,45	0,92	-1,28	0,38	0,23	0,38	0,36	0,46	0,61
aquaporin	-1,56	-3,11	-2,72	-2,55	-4,27	-3,99	-4,77	-2,41	-2,97	-3,25	-2,97	-3,34	-2,79	-3,24	-2,30	-1,31	-1,65	-2,60	-3,06	-3,94	-0,65	-0,59	-0,68	-0,66	-0,56	-0,49
14-3-3 protein	-0,41	-0,69	-1,33	-0,64	-1,13	-1,24	-1,04	-0,20	-0,23	-0,18	-0,18	-0,56	-0,33	-0,41	0,13	0,21	-0,07	-0,53	-0,71	-1,43	-0,65	-0,27	-0,71	-0,53	-0,52	-0,76
ubiquitin	-0,21	-0,42	-0,09	-1,22	-0,62	-1,07	-0,90	0,19	-0,11	-0,14	-0,06	0,04	0,09	-0,18	0,28	-0,21	-0,61	-0,65	-1,09	-1,06	-0,80	-0,52	-0,83	-0,52	-0,58	-0,86
60S ribosomal protein L14	-0,07	-0,22	-0,59	-0,67	-0,98	-1,08	-1,36	-0,02	-0,29	0,15	0,04	-0,55	-0,21	-0,79	0,08	-0,08	-0,21	-0,49	-0,77	-1,28	-0,80	-0,47	-0,81	-0,66	-0,61	-0,79
P. Taeda EST CF668373	0,23	-0,66	-1,68	-2,17	-2,20	-2,26	-2,63	0,81	1,08	0,65	0,94	0,86	0,83	0,27	0,97	0,49	-0,47	-1,53	-2,41	-2,45	-0,82	-0,43	-0,86	-0,60	-0,63	-0,96
P. Taeda EST BE241143	0,48	0,26	0,58	0,28	-0,19	-0,11	-0,38	0,27	0,70	0,82	-0,10	0,41	0,36	0,17	0,76	0,54	-0,17	-0,01	0,27	-0,20	-0,65	-0,54	-0,64	-0,56	-0,49	-0,63
acid phosphatase	0,76	-0,61	-0,47	-0,24	-1,62	-2,03	-2,46	1,30	1,05	0,16	0,17	-0,01	-0,01	-0,14	0,99	0,54	-0,76	-0,94	-1,89	-2,35	-0,90	-0,56	-0,88	-0,61	-0,52	-0,86
calmodulin	0,09	-0,42	-0,50	-0,26	-0,46	-1,09	-1,20	0,11	0,35	-0,15	0,11	0,18	0,05	-0,34	0,01	-0,02	-0,07	-0,27	-0,79	-1,41	-0,79	-0,54	-0,81	-0,59	-0,46	-0,84
ubiquitin/ribosomal protein 27a	-0,63	-0,96	-1,33	-1,41	-1,43	-2,23	-1,94	-0,43	-0,30	-0,66	-0,58	-1,03	-0,74	-1,18	-0,72	-0,55	-0,59	-0,74	-1,46	-2,15	-0,81	-0,50	-0,83	-0,67	-0,62	-0,82
dehydrin 1	0,43	-0,28	-0,86	*	-0,45	-0,50	-0,56	0,63	1,36	1,71	0,85	0,50	0,21	0,66	1,21	1,18	0,69	0,28	-0,36	-0,90	-0,60	-0,31	-0,66	-0,47	-0,48	-0,79
40S ribosomal protein S5	0,30	-0,08	-0,31	-0,35	*	-0,77	-0,69	0,23	0,22	0,09	0,21	-0,55	-0,15	-0,25	0,27	0,36	-0,13	-0,17	-0,44	-1,05	-0,81	-0,32	-0,83	-0,50	-0,48	-0,71
ubiquitin/ribosomal protein L40	-0,27	-0,82	-0,79	-1,03	-1,40	-1,51	-1,30	0,01	-0,08	-0,40	-0,10	-0,47	-0,41	-0,79	-0,02	0,08	-0,10	-0,83	-1,19	-1,13	-0,80	-0,58	-0,83	-0,66	-0,69	-0,83
ribosomal protein S7	0,10	-0,29	-0,11	-0,61	-0,44	-0,74	-0,76	0,14	0,21	0,61	0,03	-0,14	0,23	-0,05	0,30	0,09	0,05	-0,31	-0,58	-0,66	-0,76	-0,60	-0,82	-0,63	-0,64	-0,88
alpha tubulin 1	1,09	0,63	0,47	0,51	-0,86	-0,97	-1,59	1,67	2,10	1,60	*	1,26	1,66	1,19	2,07	1,82	0,94	0,50	-0,42	-0,77	-0,84	-0,65	-0,85	-0,74	-0,67	-0,93
ribosomal S26	-0,01	-0,67	-0,41	-0,99	-1,76	-2,21	-1,98	0,10	0,08	-0,05	-0,45	-0,29	-0,34	-0,64	0,63	0,14	-0,31	-0,86	-1,41	-1,93	-0,87	-0,68	-0,90	-0,71	-0,67	-0,85
xyloglucan endotransglycosylas	1,28	-0,09	0,30	-0,28	-2,02	-2,15	-2,09	1,56	1,71	1,08	1,24	1,16	0,71	0,05	1,91	1,53	1,01	-0,50	-0,36	-2,15	-0,85	-0,58	-0,86	-0,71	-0,69	-0,87
ribosomal S11	-0,07	-0,49	-1,03	-0,77	-1,16	-1,12	-1,71	0,02	-0,18	-0,53	-0,24	-0,32	0,03	-0,88	-0,72	-0,10	-0,14	-0,91	-1,34	-1,25	-0,78	-0,53	-0,80	-0,61	-0,54	-0,80
P. Taeda EST CF389725	0,94	0,36	0,61	-0,01	-0,03	-0,56	-0,74	1,18	0,88	0,61	0,80	0,63	0,63	0,40	1,19	1,10	0,94	0,11	0,15	-0,65	-0,87	-0,59	-0,87	-0,70	-0,64	-0,82
SER diff -25 / 10	3,55	4,99	6,41	7,81	8,14	8,80	9,43	3,66	4,71	6,37	6,03	5,91	6,23	5,82	4,66	5,85	6,79	7,04	7,88	8,47	1,00	0,45	0,96	0,67	0,64	0,80
RER diff -5 / 10	5,38	7,41	3,93	5,89	6,89	8,48	8,40	6,03	*	5,47	6,94	5,11	5,36	6,69	*	5,64	4,95	5,94	8,05	5,85	0,45	1,00	0,51	0,63	0,49	0,48
SER diff -15 / 10	3,90	5,91	7,26	8,94	8,82	10,03	9,69	5,81	5,57	6,90	6,65	6,51	6,40	6,05	5,31	5,87	6,99	7,84	9,29	9,41	0,96	0,51	1,00	0,65	0,65	0,82
CI / 300	0,00	0,02	0,03	0,28	0,57	0,81	1,05	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,67	0,63	0,65	1,00	0,88	0,71
CI pfn / 100	0,00	0,05	0,05	0,74	0,88	0,72	0,71	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,64	0,49	0,65	0,88	1,00	0,70
RDL (=day length /-4)	-3,25	-3,00	-2,75	-2,50	-2,25	-2,00	-1,85	-3,75	-3,75	-3,75	-3,75	-3,75	-3,75	-3,75	-3,50	-3,25	-3,00	-2,75	-2,50	-2,25	0,80	0,48	0,82	0,71	0,70	1,00

Table 3.6: Expression of candidate marker genes in the location experiment and their correlation to physiological and environmental characteristics. Columns contain: gene name, sample week number of the various experiments, and Pearson correlations of the expression profile to the corresponding physiological and environmental parameters of the samples

Table 3	A70 in Scotland						A70 in Denmark							A70 in Sweden			Pearson correlation to physiology				
week number	39	41	43	45	47	51	39	41	43	45	47	49	51	38	40	42	SER-25	RER-5	CI	CI pfn	RDL
no blast match, contig 52	-0,23	0,02	1,35	2,49	2,79	3,66	-0,72	0,47	2,45	2,24	3,22	3,32	4,00	-0,90	0,12	1,78	0,67	0,76	0,61	0,58	0,97
purple acid phosphatase	-0,20	-0,44	0,47	1,48	1,53	1,97	-0,51	-1,03	1,62	1,13	2,06	2,10	1,68	-1,07	-0,33	1,96	0,64	0,70	0,55	0,61	0,87
metallothionein	-0,45	-0,45	0,42	2,17	1,98	3,00	-2,42	*	0,91	0,91	2,10	2,89	3,35	-1,45	-0,71	1,53	0,58	0,76	0,64	0,62	0,94
transcription factor E2F	-0,01	0,11	1,37	2,62	3,52	2,45	*	*	*	1,87	2,91	2,99	2,37	-0,41	0,67	3,07	0,71	0,77	0,46	0,53	0,84
P. Taeda EST unigene seq C_C	-1,36	-1,10	-0,84	2,07	2,21	2,77	-1,21	-1,18	0,32	0,11	1,05	2,30	2,97	-1,48	-1,63	0,50	0,55	0,83	0,62	0,57	0,93
intracellular pathogenesis-relate	-0,08	-0,69	0,68	1,91	1,92	2,95	-1,68	-1,31	1,53	1,17	1,94	2,40	3,02	-1,23	-0,97	1,10	0,56	0,70	0,59	0,56	0,94
no blast match, contig 50	-0,97	-1,24	-0,82	0,83	0,76	2,61	-1,64	-1,00	0,04	-0,14	1,42	2,17	3,52	-1,02	-1,17	0,38	0,61	0,85	0,80	0,74	0,91
ELIP	1,92	1,31	0,85	2,86	3,86	6,45	0,86	-0,06	3,33	3,09	4,04	4,95	4,49	0,22	0,82	3,35	0,62	0,77	0,60	0,62	0,88
ser proteinase inhibitor	-0,70	-0,52	-0,65	1,75	1,57	2,78	-0,81	-0,52	0,89	0,82	1,73	2,78	3,35	-0,90	-0,57	1,21	0,66	0,86	0,74	0,71	0,93
LEA protein	-0,50	-0,60	0,10	1,11	0,98	1,47	-1,58	-0,72	0,20	0,14	1,21	1,82	2,46	-1,37	0,33	0,85	0,51	0,80	0,75	0,74	0,91
epoxide hydrolase	-1,12	-0,65	0,34	*	2,26	2,96	-1,61	-2,42	1,77	1,71	2,58	2,82	2,58	-2,22	0,06	2,58	0,65	0,78	0,62	0,68	0,88
CAAT binding factor subunit	-0,25	0,11	1,38	1,98	2,06	2,37	-0,35	-0,83	1,45	1,03	1,76	2,33	2,41	-0,74	-0,28	1,82	0,54	0,75	0,58	0,58	0,91
defensin (gamma thionin family)	0,98	1,15	1,39	3,64	2,96	3,97	0,28	0,58	3,13	2,32	3,07	3,24	4,33	-0,32	0,79	1,43	0,55	0,66	0,53	0,46	0,93
dehydrin dhn5	-0,76	-0,62	1,08	2,07	2,21	3,00	-1,19	-2,05	1,88	1,64	2,52	2,58	2,60	-1,77	0,16	2,56	0,55	0,74	0,56	0,61	0,88
dehydrin like	-0,17	0,77	1,27	1,91	2,31	3,14	-1,59	-1,01	2,20	1,98	2,59	2,93	2,40	-2,15	0,62	2,33	0,53	0,65	0,51	0,55	0,88
dehydrin dhn3 or dhn 7	0,06	0,49	1,70	2,71	2,73	3,21	-0,38	-0,72	2,41	2,17	3,05	3,00	3,13	-0,76	0,90	2,68	0,55	0,73	0,55	0,59	0,90
LP-3 like A	1,19	1,40	1,88	2,28	2,34	3,09	-0,24	-0,05	2,16	2,10	2,66	2,91	2,78	-0,34	1,10	2,31	0,49	0,65	0,54	0,57	0,89
aluminum induced protein	-0,29	-1,25	0,28	-0,44	-0,03	0,77	-0,86	-0,14	0,21	0,13	-0,06	0,09	0,53	-0,24	-1,95	-0,82	0,51	0,34	0,29	0,16	0,62
LP-3 like B	-0,38	0,01	1,74	2,15	2,49	2,79	-1,19	-1,97	1,85	1,35	2,35	2,99	2,55	-1,46	-0,49	2,10	0,50	0,69	0,53	0,55	0,89
thaumatin-like	-1,08	-1,41	-0,13	1,56	1,23	1,74	-1,04	-1,20	1,25	0,51	0,65	1,98	1,46	-1,67	-1,38	0,79	0,62	0,69	0,53	0,52	0,90
LP-3 like C	-0,66	-0,54	1,57	2,19	2,42	2,57	-2,12	-3,10	1,35	0,59	2,16	2,92	2,50	-2,05	-1,07	2,41	0,44	0,68	0,54	0,57	0,85
pinosylvin synthase	-0,44	-0,73	-0,42	0,26	0,37	1,97	-1,49	-0,69	-0,28	-0,31	-0,17	1,32	0,36	-1,23	-1,14	-0,24	0,43	0,67	0,50	0,50	0,86
LEA protein	0,46	0,97	-0,05	0,95	1,05	1,39	0,86	0,78	1,78	1,69	2,03	1,66	1,65	0,20	1,48	2,08	0,72	0,60	0,55	0,66	0,54
LP-3 like D	0,45	0,98	2,33	2,52	2,74	2,46	-1,47	-1,06	1,30	1,62	2,50	2,93	2,40	-1,00	0,81	3,17	0,35	0,62	0,50	0,56	0,80
sucrose synthase	-1,26	-0,89	-0,23	0,41	0,64	0,86	-1,65	-2,05	-0,01	-0,49	0,23	1,07	0,24	-0,97	-0,82	0,62	0,38	0,76	0,53	0,60	0,82
beta-1,3-glucanase	0,76	0,01	0,59	1,68	1,74	2,48	-0,66	-0,31	0,65	0,75	1,00	2,28	2,30	-0,52	-0,03	1,48	0,46	0,74	0,66	0,64	0,89
malate dehydrogenase	0,89	0,73	1,54	2,32	2,29	2,10	-0,45	-0,72	1,30	1,71	1,63	1,90	1,79	-0,51	0,69	1,58	0,30	0,54	0,37	0,39	0,82
LEA protein	0,85	-0,46	1,82	1,41	1,89	3,15	-2,28	-1,39	-0,17	0,19	0,43	0,69	0,99	-1,21	-1,42	-0,59	0,09	0,35	0,21	0,16	0,73
P. Taeda EST unigene seq C_C	-1,18	-0,79	-0,88	0,93	0,63	1,67	-1,49	-1,78	-0,74	-0,35	-0,31	1,32	-0,26	-1,70	-1,14	0,13	0,34	0,70	0,42	0,48	0,83
proline rich protein	2,16	1,16	1,89	1,34	1,58	2,41	0,44	0,79	0,82	0,20	1,83	2,48	1,92	-0,72	0,29	1,82	0,35	0,43	0,49	0,51	0,64
class IV chitinasse	-0,17	-0,01	2,03	1,91	1,92	2,01	-1,40	-1,35	0,52	0,23	0,57	1,47	1,12	-1,38	-1,21	1,19	0,25	0,50	0,34	0,33	0,78
PR10 protein	0,83	1,89	3,55	4,03	3,87	3,14	1,53	1,72	3,35	3,35	3,07	2,85	2,88	1,71	1,74	3,73	0,35	0,49	0,21	0,25	0,70
dehydrin dhn2	0,65	0,26	0,96	-0,11	0,29	0,53	1,09	0,37	0,91	0,84	0,92	0,65	0,11	0,40	0,96	1,62	0,24	-0,13	0,01	0,22	-0,24
aquaporin	-2,64	-2,91	-2,82	-3,34	-3,23	-3,06	-1,59	-1,91	-2,57	-2,16	-2,57	-2,64	-2,92	-0,81	-0,95	-0,66	-0,14	-0,15	-0,03	0,11	-0,62
14-3-3 protein	-0,01	-0,52	-0,94	-1,47	-1,09	-0,52	0,36	0,09	-0,74	-0,49	-0,97	-1,04	-0,98	0,03	-0,44	-0,55	-0,18	-0,45	-0,32	-0,32	-0,74
ubiquitin	-0,16	-0,47	-0,59	-1,39	-1,11	-1,31	-0,03	-0,42	0,09	0,02	-0,87	-1,21	-1,25	-0,23	-0,35	-1,20	-0,21	-0,77	-0,60	-0,63	-0,72
60S ribosomal protein L14	0,05	0,00	-0,75	-0,71	-0,89	-1,21	0,83	0,13	-0,48	-0,39	-1,16	-1,02	-0,71	0,39	0,17	-0,43	-0,44	-0,60	-0,43	-0,46	-0,90
P. Taeda EST CF668373	0,05	-0,26	-1,43	-2,39	-2,75	-2,64	-0,21	0,18	-2,19	-2,16	-1,56	-1,86	-2,75	0,20	-0,09	-1,97	-0,54	-0,68	-0,46	-0,42	-0,88
P. Taeda EST BE241143	*	0,28	*	-0,34	0,15	-1,09	0,35	0,76	0,08	-0,23	-0,33	-0,58	-0,81	0,46	0,43	0,00	-0,63	-0,66	-0,64	-0,66	-0,59
acid phosphatase	1,15	0,69	-0,47	-0,73	-0,58	-0,21	0,85	0,24	-0,07	-0,36	-0,51	-0,08	-0,45	1,03	0,40	-0,40	-0,46	-0,59	-0,35	-0,36	-0,80
calmodulin	-0,22	0,14	-0,41	-1,17	-0,66	-1,27	0,19	0,00	-1,03	-1,10	-1,18	-1,47	-0,96	0,32	0,25	-0,35	-0,63	-0,54	-0,42	-0,41	-0,88
ubiquitin/ribosomal protein 27a	-0,09	-0,66	-0,70	-1,97	-1,55	-1,98	0,43	-0,07	-1,22	-1,24	-1,95	-2,16	-1,29	0,51	-0,03	-0,83	-0,51	-0,62	-0,40	-0,43	-0,92
dehydrin 1	1,42	1,02	0,96	*	*	*	0,64	0,35	*	0,29	*	*	*	0,82	2,13	0,23	-0,79	-0,47	-0,21	-0,12	-0,41
40S ribosomal protein S5	0,05	-0,01	-0,58	-0,58	-0,72	-1,07	0,67	0,22	-0,80	-0,52	-1,45	-1,21	-0,81	0,72	0,38	-0,72	-0,63	-0,60	-0,50	-0,55	-0,89
ubiquitin/ribosomal protein L40	-0,26	-0,27	-0,76	-1,58	-1,52	-1,30	0,24	0,13	-1,57	-0,91	-1,31	-2,01	-1,54	0,61	0,51	-1,03	-0,60	-0,58	-0,47	-0,46	-0,88
ribosomal protein S7	-0,39	-0,02	-1,11	*	-0,88	-1,93	0,17	0,21	-0,64	-0,91	-1,69	-1,34	-1,55	0,68	0,36	-0,14	-0,73	-0,62	-0,81	-0,81	-0,83
alpha tubulin 1	0,85	0,34	-0,33	-0,95	-0,81	-1,65	1,20	1,51	-0,12	-0,58	-0,86	-1,50	-0,86	1,76	1,07	-0,27	-0,51	-0,69	-0,49	-0,50	-0,94
ribosomal S26	0,01	-0,42	-0,77	-1,67	-1,69	-1,87	0,64	0,22	-1,28	-1,07	-1,37	-1,66	-1,66	0,79	0,20	-0,64	-0,50	-0,64	-0,44	-0,41	-0,94
xyloglucan endotransglycosylas	2,24	1,54	0,27	*	0,75	*	1,07	0,98	-0,92	-0,90	-0,55	-0,09	0,74	1,61	1,25	-0,30	-0,73	-0,38	-0,22	-0,31	-0,60
ribosomal S11	-0,17	-0,16	-0,42	-1,57	-1,46	-1,66	0,98	-0,06	-0,56	*	-1,50	-1,64	-1,46	1,13	0,48	-0,91	-0,73	-0,51	-0,73	-0,80	-0,73
P. Taeda EST CF389725	1,22	0,85	0,28	-0,06	-0,04	-0,82	0,63	1,09	*	-0,11	-0,85	-0,83	-0,30	0,91	0,90	-0,12	-0,69	-0,82	-0,58	-0,65	-0,90
SER diff -25 / 10	5,59	5,59	5,82	5,53	7,01	8,02	7,97	7,99	8,95	9,05	9,41	9,63	9,78	4,76	4,94	8,58	1,00	0,62	0,60	0,59	0,63
RER diff -5 / 10	2,97	5,09	4,53	5,78	7,34	8,27	5,30	*	4,58	5,75	6,89	8,03	8,94	4,88	5,60	7,40	0,62	1,00	0,80	0,80	0,80
CI / 300	0,00	0,00	0,00	0,00	0,07	0,92	0,04	0,04	0,06	0,11	0,71	1,67	2,72	0,04	0,42	1,39	0,60	0,80	1,00	0,94	0,60
CI pfn / 100	0,00	0,00	0,01	0,00	0,19	1,60	0,11	0,00	0,07	0,16	1,78	2,90	3,13	0,12	1,14	2,92	0,59	0,80	0,94	1,00	0,57
RDL (=day length /-4)	-3,01	-2,75	-2,48	-2,23	-2,01	-1,75	-3,01	-2,76	-2,49	-2,24	-2,02	-1,86	-1,77	-3,18	-2,86	-2,55	0,63	0,80	0,60	0,57	1,00

Tabel 3.7: Expression of candidate marker genes in the provenance experiment and their correlation to physiological and environmental characteristics. Columns contain: gene name, sample week number of the various experiments, and Pearson correlations of the expression profile to the corresponding physiological and environmental parameters of the samples

Table 4	A70 provenance in Denmark							Lindas provenance in Denmark						A70 provenance in Sweden						Amsele provenance in Sweden							Pearson correlation to physiology				
week number	39	41	43	45	47	49	51	39	41	43	45	49	51	38	40	42	44	46	48	36	38	40	42	44	46	48	SER-25	RER-5	CI	CI pfn	RDL
no blast match, contig 52	-0,72	0,47	2,45	2,24	3,22	3,32	4,00	1,17	1,14	1,84	3,38	3,37	2,95	-0,66	0,90	2,19	2,62	2,78	2,14	0,23	1,68	3,42	2,76	3,54	3,16	3,02	0,87	0,52	0,53	0,66	0,76
purple acid phosphatase	-0,51	-1,03	1,62	1,13	2,06	2,10	1,68	0,10	0,41	0,52	1,95	2,13	2,03	-0,64	0,83	2,05	1,58	1,31	0,68	0,01	1,69	2,27	2,70	2,80	2,85	1,95	0,76	0,59	0,45	0,65	0,56
metallothionein	-2,42	*	0,91	0,91	2,10	2,89	3,35	-1,31	-0,24	-0,66	3,04	3,56	2,85	-2,07	-0,48	2,68	2,12	1,01	*	0,27	1,65	2,08	4,17	3,56	2,97	0,87	0,72	0,60	0,49	0,69	0,61
transcription factor E2F	*	*	*	1,87	2,91	2,99	2,37	0,61	*	0,14	2,47	3,35	2,72	-0,56	1,77	3,24	1,88	1,90	1,24	0,88	2,63	2,76	*	3,53	3,60	2,10	0,64	0,55	0,31	0,56	0,47
P. Taeda EST unigene seq C_C	-1,21	-1,18	0,32	0,11	1,05	2,30	2,97	-1,27	-1,38	-1,22	1,96	2,93	2,72	-1,43	-1,46	0,86	0,39	0,66	-0,20	-0,84	0,62	1,25	2,83	2,84	2,82	2,05	0,80	0,59	0,55	0,72	0,67
intracellular pathogenesis-relate	-1,68	-1,31	1,53	1,17	1,94	2,40	3,02	0,11	0,74	-0,29	1,81	2,20	2,07	-0,87	0,30	1,29	1,83	1,35	1,30	-1,07	1,18	0,98	2,39	2,99	2,85	*	0,81	0,62	0,61	0,73	0,75
no blast match, contig 50	-1,64	-1,00	0,04	-0,14	1,42	2,17	3,52	-1,13	-0,74	-0,76	1,89	4,10	3,57	-1,01	-1,05	1,57	2,39	1,45	1,41	-1,17	-0,28	1,41	4,92	4,64	4,20	3,18	0,76	0,74	0,69	0,84	0,71
ELIP	0,86	-0,06	3,33	3,09	4,04	4,95	4,49	0,83	*	0,55	4,67	5,25	3,72	0,20	1,26	5,28	2,34	1,17	0,66	0,46	3,13	5,82	6,87	4,23	2,95	*	0,68	0,36	0,14	0,45	0,37
ser proteinase inhibitor	-0,81	-0,52	0,89	0,82	1,73	2,78	3,35	0,37	0,24	-0,39	1,57	2,87	2,88	-0,19	-0,49	1,58	2,12	2,71	2,35	-0,98	0,26	2,05	2,67	2,26	1,59	2,04	0,76	0,71	0,70	0,84	0,81
LEA protein	-1,58	-0,72	0,20	0,14	1,21	1,82	2,46	-1,31	0,13	-0,47	1,05	2,05	2,33	-1,36	-0,11	0,94	1,46	1,49	1,20	-0,40	0,78	1,15	2,16	2,21	2,23	2,12	0,78	0,77	0,72	0,84	0,76
epoxide hydrolase	-1,61	-2,42	1,77	1,71	2,58	2,82	2,58	-0,26	-0,54	-0,39	2,00	3,31	2,41	-0,99	0,96	2,64	1,53	1,08	0,34	-0,51	1,35	1,19	1,69	1,76	1,14	0,61	0,64	0,38	0,32	0,58	0,58
CAAT binding factor subunit	-0,35	-0,83	1,45	1,03	1,76	2,33	2,41	-0,15	0,12	0,52	2,28	2,22	1,93	-0,19	0,69	1,75	1,62	1,17	-0,10	0,31	2,24	2,32	3,45	3,38	2,89	1,24	0,71	0,51	0,31	0,55	0,42
defensin (gamma thionin family)	0,28	0,58	3,13	2,32	3,07	3,24	4,33	0,54	0,73	1,16	3,25	3,47	3,75	0,46	1,41	1,86	1,23	1,16	0,86	1,59	2,43	3,81	3,93	2,36	2,47	2,61	0,68	0,24	0,20	0,41	0,45
dehydrin dhn5	-1,19	-2,05	1,88	1,64	2,52	2,58	2,60	-0,25	-0,24	-0,11	2,42	2,70	2,13	-0,79	1,27	2,34	1,71	1,48	0,81	-0,33	1,89	2,60	2,97	2,88	2,88	1,62	0,69	0,55	0,41	0,63	0,57
dehydrin like	-1,59	-1,01	2,20	1,98	2,59	2,93	2,40	-0,11	0,17	0,27	3,02	3,01	2,19	-0,70	1,17	2,96	1,31	1,05	0,75	-0,23	2,20	3,43	2,94	2,55	2,74	1,29	0,67	0,41	0,27	0,51	0,49
dehydrin dhn3 or dhn 7	-0,38	-0,72	2,41	2,17	3,05	3,00	3,13	0,92	1,00	1,25	3,14	2,97	2,53	-0,02	1,81	2,68	1,83	1,83	1,28	1,07	2,83	2,91	3,56	3,31	3,30	1,97	0,70	0,44	0,31	0,53	0,49
LP-3 like A	-0,24	-0,05	2,16	2,10	2,66	2,91	2,78	0,76	1,11	1,15	2,99	3,01	2,54	0,18	0,96	2,70	2,15	1,49	1,13	1,27	2,32	3,00	3,38	2,87	1,98	1,62	0,71	0,39	0,23	0,50	0,45
aluminum induced protein	-0,86	-0,14	0,21	0,13	-0,06	0,09	0,53	0,02	0,54	-0,06	0,55	0,82	0,93	-1,28	-1,22	-0,90	-0,52	0,01	0,03	-0,16	0,35	-0,09	-0,03	0,53	1,02	1,08	0,75	0,28	0,43	0,32	0,57
LP-3 like B	-1,19	-1,97	1,85	1,35	2,35	2,99	2,55	-0,22	-0,15	0,34	2,99	3,03	2,39	-0,53	1,01	2,64	2,16	2,28	1,01	0,71	2,59	2,82	3,08	3,33	2,93	1,55	0,67	0,57	0,41	0,62	0,51
thaumatin-like	-1,04	-1,20	1,25	0,51	0,65	1,98	1,46	-0,50	0,12	-0,68	2,73	2,04	1,32	-0,72	0,15	1,93	2,13	1,76	1,03	1,09	2,44	3,22	3,41	2,55	1,69	1,33	0,54	0,58	0,35	0,53	0,32
LP-3 like C	-2,12	-3,10	1,35	0,59	2,16	2,92	2,50	-0,99	*	-0,50	2,36	3,24	2,22	-1,52	0,54	2,62	1,99	2,18	0,80	-0,11	2,45	3,08	5,07	3,69	3,58	1,56	0,70	0,66	0,43	0,66	0,48
pinosylvin synthase	-1,49	-0,69	-0,28	-0,31	-0,17	1,32	0,36	-0,87	-0,78	-0,27	1,23	2,18	0,71	-1,38	-1,70	0,30	0,45	0,40	0,13	-0,78	-0,52	-0,60	1,86	1,15	0,45	0,33	0,77	0,48	0,49	0,67	0,67
LEA protein	0,86	0,78	1,78	1,69	2,03	1,66	1,65	1,01	1,13	1,31	1,75	1,44	1,10	0,36	1,14	2,28	0,63	0,17	-0,13	0,18	1,36	1,86	1,91	1,13	0,62	0,12	0,37	-0,22	-0,43	-0,10	0,00
LP-3 like D	-1,47	-1,06	1,30	1,62	2,50	2,93	2,40	-0,64	-0,16	0,49	2,57	2,29	1,88	-1,03	0,41	3,44	1,89	1,38	0,62	-0,51	1,67	*	3,15	2,73	2,32	0,48	0,70	0,47	0,35	0,62	0,57
sucrose synthase	-1,65	-2,05	-0,01	-0,49	0,23	1,07	0,24	-0,78	*	-1,00	0,43	1,38	0,80	-1,16	-0,48	1,34	0,45	0,51	0,19	-0,63	0,22	0,85	2,70	2,07	1,55	0,60	0,65	0,72	0,50	0,73	0,48
beta-1,3-glucanase	-0,66	-0,31	0,65	0,75	1,00	2,28	2,30	0,26	0,21	0,30	1,69	2,13	1,29	-0,09	-0,22	1,13	2,61	2,46	1,74	0,35	0,49	1,64	4,33	2,09	0,91	1,71	0,64	0,66	0,56	0,73	0,57
malate dehydrogenase	-0,45	-0,72	1,30	1,71	1,63	1,90	1,79	0,34	1,07	0,62	1,69	1,66	2,27	0,27	1,48	2,07	1,77	1,12	1,25	1,11	2,26	2,68	2,73	2,65	2,44	1,90	0,58	0,63	0,39	0,57	0,39
LEA protein	-2,28	-1,39	-0,17	0,19	0,43	0,69	0,99	-1,05	-0,77	-0,79	1,26	1,17	1,42	-1,24	-0,49	0,28	0,83	0,97	1,13	-1,60	-0,09	0,31	1,66	1,25	0,79	0,38	0,74	0,68	0,63	0,77	0,76
P. Taeda EST unigene seq C_C	-1,49	-1,78	-0,74	-0,35	-0,31	1,32	-0,26	-1,64	-2,63	-1,34	0,11	1,29	-0,42	-1,40	-1,62	0,15	0,48	-0,08	-0,78	-1,49	-1,29	-1,97	1,87	*	*	-1,78	0,51	0,34	0,30	0,59	0,50
proline rich protein	0,44	0,79	0,82	0,20	1,83	2,48	1,92	0,87	0,94	1,02	1,20	2,05	1,80	0,17	1,07	1,87	3,04	2,98	1,71	0,79	1,44	1,20	1,72	2,96	2,53	1,93	0,59	0,80	0,73	0,84	0,64
class IV chitinasse	-1,40	-1,35	0,52	0,23	0,57	1,47	1,12	-0,92	-0,71	-1,30	2,05	1,52	1,07	0,18	0,65	2,17	2,85	2,61	2,35	1,10	1,68	2,53	3,70	3,32	3,27	2,70	0,41	0,81	0,64	0,73	0,42
PR10 protein	1,53	1,72	3,35	3,35	3,07	2,85	2,88	3,23	3,33	2,33	4,01	2,37	2,54	3,19	4,13	4,29	4,42	4,40	4,03	5,10	5,43	5,23	4,32	4,65	4,25	3,45	-0,09	0,38	0,13	0,15	-0,20
dehydrin dhn2	1,09	0,37	0,91	0,84	0,92	0,65	0,11	1,02	0,45	1,39	0,58	-0,25	-0,70	1,03	1,39	1,27	0,83	0,25	-0,01	0,92	1,44	1,03	0,30	0,81	0,37	-0,26	-0,51	-0,50	-0,67	-0,59	-0,70
aquaporin	-1,59	-1,91	-2,57	-2,16	-2,57	-2,64	-2,92	-1,83	-2,54	-1,42	-2,75	-3,60	-2,83	-0,42	-0,64	-0,95	-2,18	-2,16	-2,48	-1,95	-2,24	-2,61	-3,85	-1,73	-1,40	-2,39	-0,64	-0,20	-0,21	-0,30	-0,44
14-3-3 protein	0,36	0,09	-0,74	-0,49	-0,97	-1,04	-0,98	-0,18	-0,56	-0,14	-0,98	-1,26	-1,04	-0,08	-0,52	-0,40	-0,88	-0,90	-1,41	-0,52	-0,80	-1,52	-1,34	-1,79	-1,12	-1,47	-0,62	-0,71	-0,63	-0,69	-0,59
ubiquitin	-0,03	-0,42	0,09	0,02	-0,87	-1,21	-1,25	-0,33	0,06	-0,06	-0,79	-1,38	-0,96	-0,01	-0,68	-1,06	-0,97	-0,83	-0,98	0,21	-0,38	-0,80	-1,41	-1,58	-2,19	-1,21	-0,59	-0,82	-0,72	-0,85	-0,66
60S ribosomal protein L14	0,83	0,13	-0,48	-0,39	-1,16	-1,02	-0,71	0,13	-0,34	0,29	-0,82	-1,08	-0,83	0,06	-0,47	-0,58	*	-2,09	-1,83	-0,17	-0,53	-1,58	-1,45	-1,10	-1,28	-0,74	-0,48	-0,75	-0,65	-0,71	-0,59
P. Taeda EST CF668373	-0,21	0,18	-2,19	-2,16	-1,56	-1,86	-2,75	-0,27	-0,14	-0,93	-1,74	-1,86	-2,40	-0,47	-0,63	-2,98	-0,69	-1,19	-0,89	-0,44	-2,53	-3,71	-4,19	-1,94	-1,66	-1,18	-0,53	-0,34	-0,14	-0,38	-0,25
P. Taeda EST BE241143	0,35	0,76	0,08	-0,23	-0,33	-0,58	-0,81	0,45	0,80	0,06	-0,91	-0,40	-0,26	0,26	0,49	-0,04	-0,43	-0,07	0,22	0,29	0,45	-0,18	*	-0,08	0,19	0,19	-0,61	-0,13	-0,25	-0,44	-0,58
acid phosphatase	0,85	0,24	-0,07	-0,36	-0,51	-0,08	-0,45	0,46	0,44	0,10	-0,61	-0,47	-0,54	0,24	0,27	-1,07	-0,76	-0,45	-0,59	1,04	-0,91	*	-2,88	-1,20	-0,59	*	-0,54	-0,60	-0,42	-0,59	-0,41
calmodulin	0,19	0,00	-1,03	-1,10	-1,18	-1,47	-0,96	-0,17	-0,08	-0,45	-1,60	-1,59	-0,88	-0,02	-0,22	-0,24	-1,21	-0,83	-0,86	0,05	-0,32	-0,72	0,68	-0,60	-0,58	-0,56	-0,54	-0,05	-0,27	-0,30	-0,66
ubiquitin/ribosomal protein 27a	0,43	-0,07	-1,22	-1,24	-1,95	-2,16	-1,29	-0,20	-0,91	-0,11	-1,69	-2,74	-1,44	0,02	-0,40	-1,08	-2,26	-1,49	-0,84	-0,61	-1,08	-2,03	-2,74	-1,97	-1,42	-0,92	-0,69	-0,52	-0,33	-0,59	-0,51
dehydrin 1	0,64	0,35	*	0,29	*	*	*	0,16	*	*	*	*	*	0,80	-0,03	-0,92	0,22	0,21	0,94	0,68	-0,75	*	*	-1,14	-1,02	*	-0,61	-0,49	-0,17	-0,39	-0,19
40S ribosomal protein S5	0,67	0,22	-0,80	-0,52	-1,45	-1,21	-0,81	0,23	-0,10	0,43	-0,63	-1,10	-0,82	0,38	-0,12	-0,95	-1,49	-1,30	-1,04	0,16	-0,25	*	-1,31	-0,94	-0,77	0,19	-0,57	-0,57	-0,47	-0,69	-0,63
ubiquitin/ribosomal protein L40	0,24	0,13	-1,57	-0,91	-1,31	-2,01	-1,54	-0,03	-1,15	0,44	-1,98	-2,31	-1,85	0,29	-0,23	-0,60	-0,80	-1,74	-1,67	-0,59	-1,03	-2,22	-3,27	-2,28	-1,89	-1,51	-0,69	-0,57	-0,47	-0,61	-0,55
ribosomal protein S7	0,17	0,21	-0,64	-0,91	-1,69	-1,34	-1,55	0,11	-0,54	-0,40	-1,25	-0,73	-1,04	-0,05	0,03	-0,66	*	-1,14	*	-0,46	-0,29	0,60	-0,16	-0,84	*	*	-0,61	-0,14	-0,54	-0,49	-0,77
alpha tubulin 1	1,20	1,51	-0,12	-0,58	-0,86	-1,50	-0,86	1,08	1,12	0,81	*	-1,69	*	1,43	1,14	-0,42	0,18	0,28	0,26	1,86	1,00	0,25	*	-0,30	-0,11	0,38	-0,75	-0,35	-0,39	-0,64	-0,76
ribosomal S26	0,64	0,22	-1,28	-1,07	-1,37	-1,66	-1,66	0,03	-0,51	-0,02	-1,03	-2,00	-1,68	0,29	0,12	-0,78	-1,29	-0,94	-1,14	-0,34	-0,81	-1,72	-1,52	-1,66	-1,61	-0,98	-0,78	-0,57	-0,51	-0,67	-0,68
xyloglucan endotransglycosylas	1,07	0,98	-0,92	-0,90	-0,55	-0,09	0,74	0,74	*	1,55	-1,39	-1,57	-0,53	1,57	1,17	-0,31	0,04	-0,13	-0,05	1,79	0,36	*	-3,86	-0,57	0,08	0,12	-0,60	-0,29	-0,19	-0,39	-0,41
ribosomal S11	0,98	-0,06	-0,56	*	-1,50	-1,64	-1,46	0,09	0,08	-0,15	-0,91	-1,64	-1,37	0,34	0,11	-1,24	-1,45	-1,09	0,09	-0,49	-0,98	*	*	-2,03	-1,42	-1,09	-0,73	-0,58	-0,47	-0,69	-0,61
P. Taeda EST CF389725	0,63	1,09	*	-0,11	-0,85	-0,83	-0,30	0,50	0,29	1,47	-0,18	-1,48	-0,42	1,13	1,16	-0,54	-0,12	-0,21	0,28	*	0,19	-0,25	*	-0,27	-0,11	-0,01	-0,68	-0,44	-0,37	-0,61	-0,60
SER diff -25 / 10	7,97	7,99	8,95	9,05	9,41	9,63	9,78	8,12	9,06	9,35	9,48	9,76	9,83	7,04	7,19	8,42	8,98	9,24	8,18	7,06	8,55	9,01	9,53	9,75	9,59	9,64	1,00	0,38	0,44	0,54	0,75
RER diff -5 / 10	5,30	*	4,58	5,75	6,89	8,03	8,94	5,99	*	5,03	5,41	8,68	8,82	6,51	7,79	8,93	9,71	10,00	9,73	5,87	7,71	8,97	9,57	10,15	9,88	9,72	0,38	1,00	0,81	0,90	0,51
CI / 300	0,04	0,04	0,06	0,11	0,71	1,67	2,72	0,04	0,04	0,06	0,11	1,67	2,72	0,04	0,42	1,39	2,40	3,44	4,56	0,01	0,04	0,42	1,39	2,40	3,44	4,56	0,44	0,81	1,00	0,89	0,78
CI pfn / 100	0,11	0,00	0,07	0,16	1,78	2,90	3,13	0,11	0,00	0,07	0,16	2,90	3,13	0,12	1,14	2,92	3,03	3,12	3,36	0,04	0,08	1,14	2,92	3,03	3,12	3,36	0,54	0,90	0,89	1,00	0,77
RDL (=day length /-4)	-3,01	-2,76	-2,49	-2,24	-2,02	-1,86	-1,77	-3,01	-2,76	-2,49	-2,24	-1,86	-1,77	-3,18	-2,86	-2,55	-2,23	-1,93	-1,67	-3,50	-3,18	-2,86	-2,55	-2,23	-1,93	-1,67	0,75	0,51	0,78	0,77	1,00

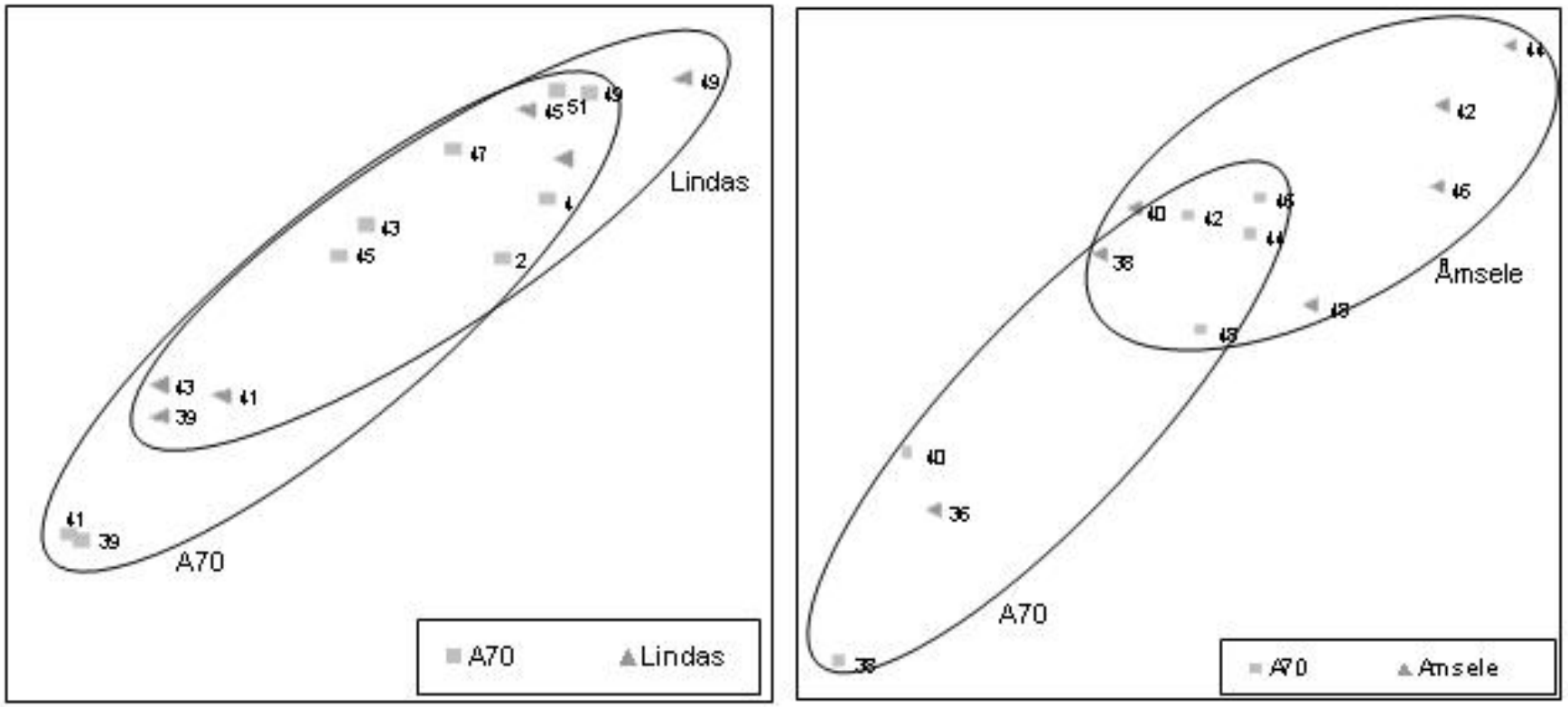


Figure 3.55. Principal component analysis of the provenance experiment. Left panel: Results from Denmark, triangles: Lindås; squares: A70. Right panel: Results from Sweden. triangles: Åmsele; squares: A70. The first two principal components explain 79.7% of the observed variance. Week numbers are also indicated.

in CI pfn), while day length declines slightly faster as well (steeper slope of RDL in Fig. 3.54A).
When comparing the cold hardening process in local and standard provenances, it can be seen that at both locations the local provenance outperforms the standard provenance from Scotland. In Denmark the local provenance Lindås precedes A70 in acquisition of cold tolerance, but in week 51 both provenances have reached similar levels of root and shoot frost hardiness (Fig 3.54B and 3.54C). In Sweden, the local provenance Åmsele already has a higher cold tolerance at the onset of the experiment (week 38) and stays ahead of A70 for the rest of the season (Fig 3.54B and C). Only in roots the standard provenance reaches a similar level of frost tolerance as the local provenance (Fig 3.54C). It seems that the non-local provenance A70 has difficulty adapting to the climatic conditions in Sweden, since shoots do not become fully frost tolerant (Fig 3.54B). Additionally, A70 seems to have difficulty maintaining the level of frost tolerance that is required to survive Swedish winters, since the SER diff -25 in week 48 in Sweden (S48) is lower than in week 46, indicating loss of frost hardiness (Fig 3.54B). This premature loss of cold tolerance in A70 in Sweden was observed in independent experiments using 1 or 2 year old seedlings during 2 consecutive seasons (data not shown).

Figure 3.55 shows a principal component analysis of the samples from the provenance experiment, based on the hybridisation patterns of the descriptive gene set. Samples are more or less placed on a diagonal, with the least cold tolerant samples on the lower left, and the more cold tolerant samples on the top right side of each panel. The A70 samples are concentrated more on the lower left side, while the local provenances samples are positioned more towards the top right of each panel, indicating their higher level of frost hardiness. Interestingly, the samples from week 48 in Sweden from both provenances show a gene expression profile in the buds that is less indicative of high cold tolerance, while only in A70 this results in a measurable physiological effect in the shoot. It is possible that after the acquisition of cold tolerance, gene expression levels reach a lower “steady state” level that under normal circumstances allows maintenance of frost hardiness. In the case of A70 shoots, this frost hardiness is prematurely lost, resulting in seedlings that do not survive frozen storage under standard conditions (pers. Comm. E.S.).

Table 3.7 shows the expression ratios of the candidate marker genes in the provenance experiment, as well as the physiological and environmental parameters of each sample. The putative markers genes show good correlation to both $SER_{diff\ -25}$ and RDL, while correlation between $SER_{diff\ -25}$ and $RER_{diff\ -5}$ is again poor.

3.9 Transcriptional profiling of beech

3.9.1 Gene expression profiles during frost tolerance development in field conditions

Data on shoot electrolyte leakage after freezing are shown in Fig. 3.56A. These data are from seedlings of the same age, field grown at two different locations, Denmark and Scotland, in the same year (2001). From the profiles it is clear that frost tolerance develops simultaneously. There is a significant decline in leakage during the period from week 37 to week 45, indicating acquiescence of frost tolerance. From week 45 onward, leakage remained low and almost constant.
Data indicating the amount of experienced cold (cold index) for both series are shown in Fig. 3.56B. In Denmark temperature declines at least two weeks earlier than in Scotland. The slope of the line connecting the data points from Denmark is steeper in general than the slope of the line from the Scotland data. So the total amount of experienced cold is higher in Denmark for our experimental period.
Fig. 3.56C shows data indicating bud-break potential for both locations. Whereas beech seedlings grown in Denmark displayed a distinct period where bud-break potential is high, seedlings grown in Scotland didn’t.
Samples from week 41 to week 04 (56) from both locations were hybridised to the microarrays against a common reference. After quality selection of hybridisation signals as described, expression ratios from 196 PCR fragments were selected for cluster

Table 3.8 Contents of clusters presented in Figure 3.58.
Genes that could be categorised in the same cluster for both one-year-old seedlings from Denmark and Scotland, planted in 2001, are listed. Genes presented in bold characters are the ones that follow the frost tolerance profile best (positively as well as negatively correlated).

Table 3.8A

Cluster	Suggested function	Clone identity	Functional category
strong increase	**ABA-inducible protein**	B1nr014	cold/drought stress
Increase	dehydrin	B1nr013	cold/drought stress
	dehydrin	B1nr033	cold/drought stress
	embryonic abundant protein, 59K - soybean	B5nr012	cold/drought stress
	embryonic abundant protein AtEm1	B5nr040	cold/drought stress
	dehydrin-like protein	B5nr083	cold/drought stress
	ABA-inducible protein	B6nr024	cold/drought stress
	dehydrin 1 - Commerson's wild potato	B6nr027	cold/drought stress
	dehydrin-like protein	B6nr059	cold/drought stress
	osmotin-like protein	B4nr079	other stresses
	protein kinase family	B5nr018	protein metabolism
	PRL1 associated protein -related	B5nr052	signalling
	eukaryotic translation initiation factor 4A-1 (eIF4A-1)	B5nr086	translation
	elongation factor 1 alpha	B6nr026	translation
	unknown	B1nr021	unknown identity
	unknown	B1nr024	unknown identity
	unknown	B1nr034	unknown identity
	unknown	B1nr035	unknown identity
	unknown	B1nr038	unknown identity
	unknown	B1nr039	unknown identity
	unknown	B1nr048	unknown identity
	unknown	B4nr049	unknown identity
	unknown	B4nr084	unknown identity
	unknown	B5nr087	unknown identity
	unknown	B6nr008	unknown identity
	unknown	B6nr038	unknown identity
	unknown	B6nr056	unknown identity
	unknown	B6nr067	unknown identity
Moderate increase	LTCOR11	B5nr078	cold/drought stress
	LTCOR11	B6nr074	cold/drought stress
	put.early light induced protein	B1nr055	others
	early light-induced protein	B6nr057	others
	probable light induced protein - Arabidopsis thaliana	B6nr061	others
	unknown	B4nr081	unknown identity
	unknown	B4nr096	unknown identity
	unknown	B5nr055	unknown identity
	unknown	B5nr060	unknown identity
	unknown	B6nr022	unknown identity
	unknown	B6nr046	unknown identity
	unknown	B6nr047	unknown identity

Table 3.8B

Cluster	Suggested function	Clone identity	Functional category
Decline	Sucrose synthase	B2nr055	carbohydrate metabolism
	cellulose synthase catalytic subunit	B2nr093	carbohydrate metabolism
	allergenic isoflavone reductase-like protein	B1nr082	defence
	chalcone synthase	B3nr078	defence
	diphenol oxidase laccase	B4nr022	defence
	arabinogalactan protein	B1nr019	growth and development
	alpha-tubulin	B2nr041	growth and development
	expansin- related	B3nr056	growth and development
	beta tubulin	B4nr043	growth and development
	GDSL-motif lipase/hydrolase protein	B1nr005	lipid metabolism
	GDSL-motif lipase/hydrolase protein	B3nr066	lipid metabolism
	polyphenol oxidase	B3nr090	other stresses
	unknown	B3nr031	unknown identity
	unknown	B1nr025	unknown identity
	unknown	B1nr027	unknown identity
	unknown	B1nr041	unknown identity
	unknown	B1nr074	unknown identity
moderate decline	2-dehydro-3-deoxyphosphoheptonate aldolase	B1nr068	carbohydrate metabolism
	xyloglucan endotransglycosylase precursor	B3nr012	carbohydrate metabolism
	glyceraldehyde-3-phosphate dehydrogenase	B4nr007	carbohydrate metabolism
	glyceraldehyde-3-phosphate dehydrogenase	B4nr012	carbohydrate metabolism
	glyceraldehyde 3-phosphate dehydrogenase	B6nr033	carbohydrate metabolism
	putative DnaJ protein	B2nr027	cold/drought stress
	low-temperature-induced protein 65	B5nr046	cold/drought stress
	hydroxymethylglutaryl-CoA reductase	B4nr010	energy metabolism
	dUTP pyrophosphatase-related protein	B4nr024	energy metabolism
	alpha-tubulin	B3nr055	growth and development
	beta tubulin	B3nr095	growth and development
	dihydrolipoamide S-acetyltransferase	B3nr025	lipid metabolism
	acyltransferase-like protein	B3nr069	lipid metabolism
	bacterial-induced peroxidase precursor	B2nr048	other stresses
	26S proteasome AAA-ATPase subunit RPT6a	B1nr080	others
	auxin-induced protein-related	B3nr093	others
	Peptide methionine sulfoxide reductase	B1nr028	protein metabolism
	thioredoxin H-type 1	B3nr029	protein metabolism
	serine/threonine-protein kinase-like protein	B5nr063	protein metabolism
	polyubiquitin	B6nr071	protein metabolism
	putative HMG-like protein	B1nr006	transcription regulation
	homeodomain-leucine zipper protein 56	B3nr079	transcription regulation
	nucleoid DNA-binding - like protein	B4nr020	transcription regulation
	putative MYB family transcription factor	B4nr023	transcription regulation
	C-repeat/DRE binding protein, putative	B5nr096	transcription regulation
	myb-like transcription factor 1	B6nr013	transcription regulation
	60S ribosomal protein L15	B4nr080	translation
	60S ribosomal protein L36	B5nr009	translation
	60S acidic ribosomal protein P2	B5nr056	translation
	unknown	B1nr004	unknown identity
	unknown	B2nr004	unknown identity
	unknown	B2nr070	unknown identity
	unknown	B2nr074	unknown identity
	unknown	B2nr083	unknown identity
	unknown	B3nr019	unknown identity
	unknown	B3nr083	unknown identity
	unknown	B4nr047	unknown identity
	unknown	B6nr003	unknown identity
	unknown	B6nr025	unknown identity
	unknown	B6nr086	unknown identity
	unknown	B1nr044	unknown identity
	unknown	B1nr056	unknown identity

analysis. In both series, seedlings grown in Denmark as well as in Scotland, hybridisation of these fragments resulted in data that could be used for further analysis. The putative identities of those clones were used for classification and the result is shown in Figure 3.57A. The population of cDNA fragments used for selection of fragments for the microarray had been enriched for cDNA clones predominantly present in the frost sensitive state as well as in the frost tolerant state. This is for instance illustrated by the high amount of fragments that show identity with genes related to cold/drought stress. This category contains genes homologous to LEA genes such as dehydrins, embryonic abundant proteins, ABA inducible genes and LTCOR homologues. Within the category of genes related to transcriptional regulation there are homologous to the C-repeat/DRE binding proteins, WRKY family members and several MYB binding factors. Among the group of genes related to growth and development there are several members of the tubulin family of genes and an expansin related homologous gene. Twelve different categories could be defined next to a large category of cDNA fragments where no significant homology was found. Cut off value for homology was e-5. Also fragments with high homology to Arabidopsis cDNA clones with unknown function were categorised in this group.
Genes that are of special interest, based on the results obtained with Scots pine, were spotted multiple times on the array and whenever possible multiple (partly) homologous clones. Among those are the ABA-inducible gene (6 different contigs), dehydrins (5 different contigs), embryonic abundant proteins (3 different contigs) and alpha- and beta-tubulins (in total 5 different contigs). Several members from the mentioned transcriptional regulation group of genes were spotted 4 times in duplicate (C-repeat/DRE and WRKY).
Data from both series were independently subjected to Hierarchical clustering (Euclidean distance). The resultant picture for the expression ratios from seedlings grown in Denmark is shown in Fig. 3.57B. It is shown that the expression of most genes, represented by PCR fragments on the microarray, changed during the examined period.
Different clusters could be distinguished (Fig. 3.58) and genes that were found to be in identical clusters for both series of microarray hybridisations are listed in Table 3.8. Besides hardly changing expression, delta 2log ratio < two, two characteristic expression profiles were found. The first one showed an increase from week 41 to week 47 followed by a decline (Fig.3.58A). Based on the magnitude of the change, three different clusters could be distinguished. Most genes found in those clusters could be categorised as being related to cold/drought stress (Table 3.8A). The second one showed stable expression until week 47 followed by a decline (Fig.3.58B). Based on the magnitude of the change, two different clusters could be distinguished. Genes found to be in those clusters could be categorised as being related to basic metabolism as well as growth and development and transcription regulation (Table 3.8B).
In general, genes that were spotted multiple times (more than twice) behaved like good replicates.
Genes found to cluster together (Euclidean distance) with normalised frost tolerance (SEL diff-15ºC (%)) values were compared in between both series. Corresponding genes are indicated in Table 3.8 (bold characters).
Figure 3.59 shows a few examples of the expression profiles of genes that are either upregulated or downregulated. This is to illustrate the fact that in general genes that are upregulated appeared to show no timing-difference in expression profile between the two series of beech seedlings whereas genes that are downregulated in general do. This difference in timing for the downregulated genes is not correlated with values for experienced cold (Fig.3.56B).
All functional categories are represented within the group defined as hardly changing genes.
To validate the results of the microarray hybridisation a selection of genes was re-profiled using real-time PCR. Figure 3.60 shows the result of this analysis on four genes in apical buds of one-year-old seedlings, grown in Denmark (2001). The expression patterns are very similar to those found with the microarray. The amplitude to the change in expression was found to be larger.

3.9.2 Expression profiles of relevant genes under controlled climate conditions

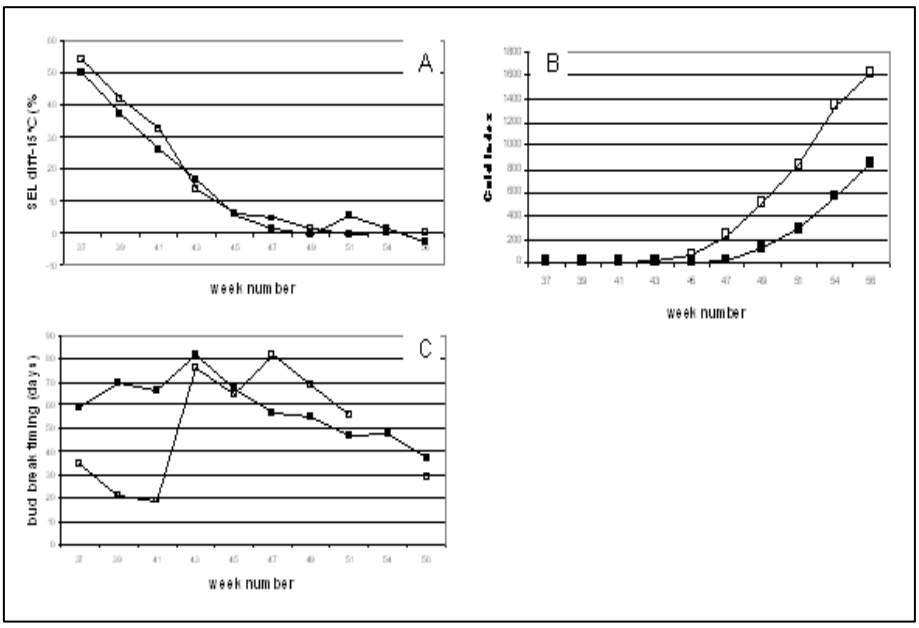


Figure 3.56 Physiological data of the one-year-old seedlings used for assessment of expression profiles during autumn and winter.
A. Frost tolerance profiles, measured as freeze induced Shoot Electrolyte Leakage percentages.
B. Experienced hours with a temperature below 4ºC.
C. Dormancy profiles, measured as bud break timing after transfer to optimal growing conditions. Open symbols represent data from seedlings planted in Denmark, 2001. Closed symbols represent data from seedlings planted in Scotland, 2001.

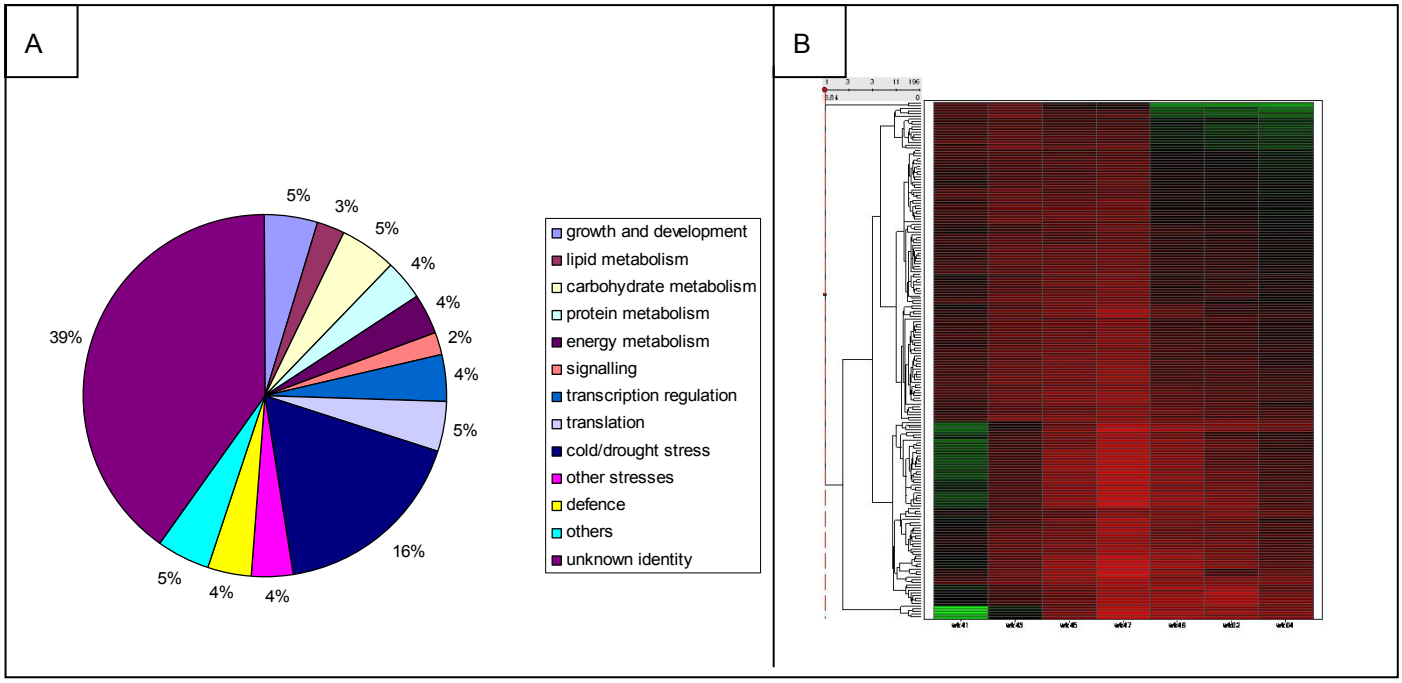


Figure 3.57
A Functional categories on the microarray.
The 196 clones that were used for cluster analysis were categorised using the publicly available MIPS database.
B Hierarchical clustering of microarray data from one-year-old seedlings planted in Denmark, 2001.

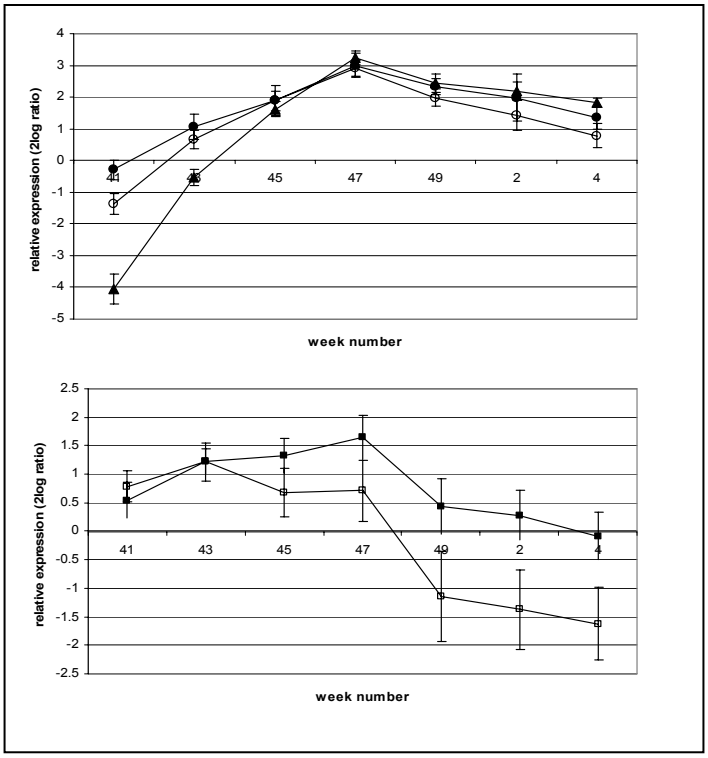
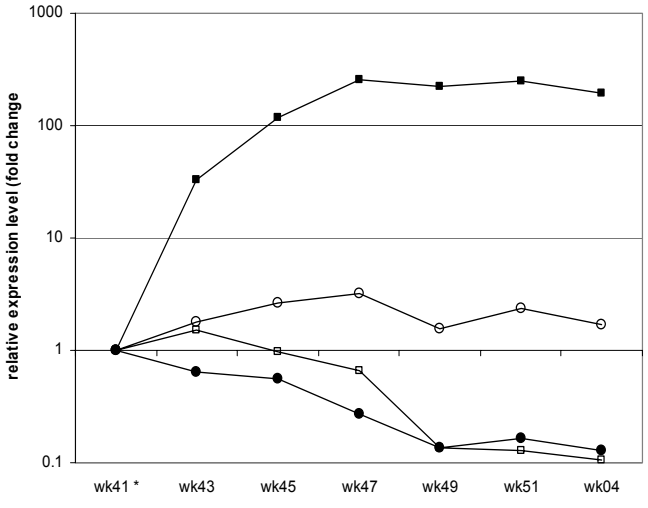


Figure 3.58 Expression profiles of one-year-old seedlings planted in Denmark, 2001.
A. Three different clusters representing genes that show increased expression. Filled circles correspond to the moderate increase class, presented in Table 3.8. Open circles correspond to the increase class, presented in Table 3.8. Filled triangles correspond to the group of ABA-inducible proteins, presented as the strong increase class in Table 3.8.
B. Two different clusters representing genes with a decrement in expression. Filled symbols correspond to the moderate decline class, presented in Table 3.8B. Open symbols correspond to the decline class, presented in Table 3.8B.



Symbol	Expression profile	Suggested function	Primersequences 5'-->3'
closed squares	increasing	ABA inducible protein	CAGGCCAAGGGCCAAACTCAGG AGCAGCCCCTTGTGCCTTAGCC
open squares	decreasing	alpha tubulin	TCCCGCATTGACCACAAGTTCG TCCAGGGCAGCAAGGTCCTCAC
closed circles	decreasing	unknown	CGGCCATGGATGTCGTTTACGC CACCACCACCACCATTTGCAGAC
open circles	constant	protein kinase	GATCCAATTGTGATGGCGAGCTG ACCAGCAACATCGCCCATACTG

Figure 3.60 Technical validation of expression profiles.
Realtime RT-PCR data of a selection of genes measured in one-year-old seedlings planted in Denmark, 2001.

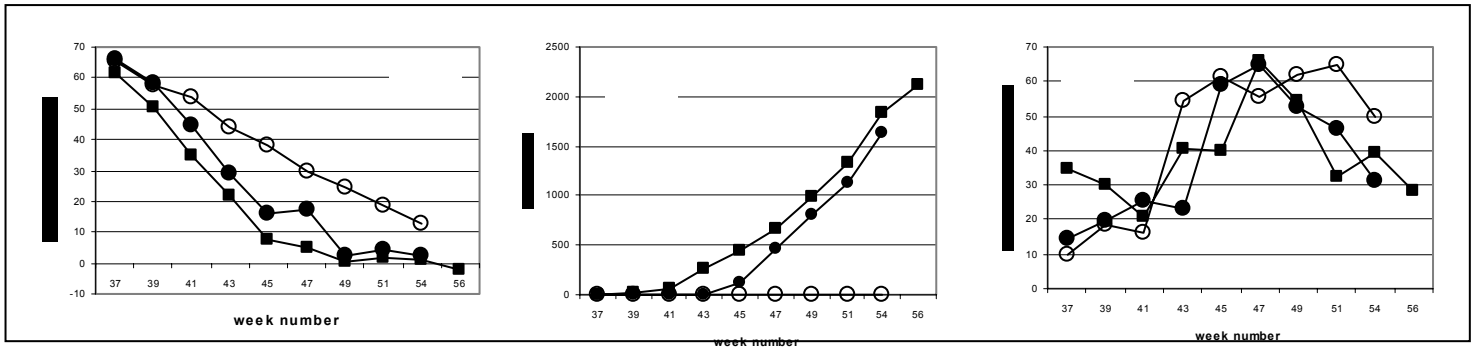


Figure 3.61 Physiological data of seedlings that were planted under controlled climate conditions compared to the situation in the field.
A. Frost tolerance profiles, measured as freeze induced Shoot Electrolyte Leakage percentages.
B. Experienced hours with a temperature below 4ºC.
C. Dormancy profiles, measured as bud break timing after transfer to optimal growing conditions. Open circles represent data from the climate where temperature and daylength were kept constant from week 33, 2002 on. Closed circles are data from the climate where both temperature and daylength gradually decline. Squares are data from the field (Denmark, 2002).

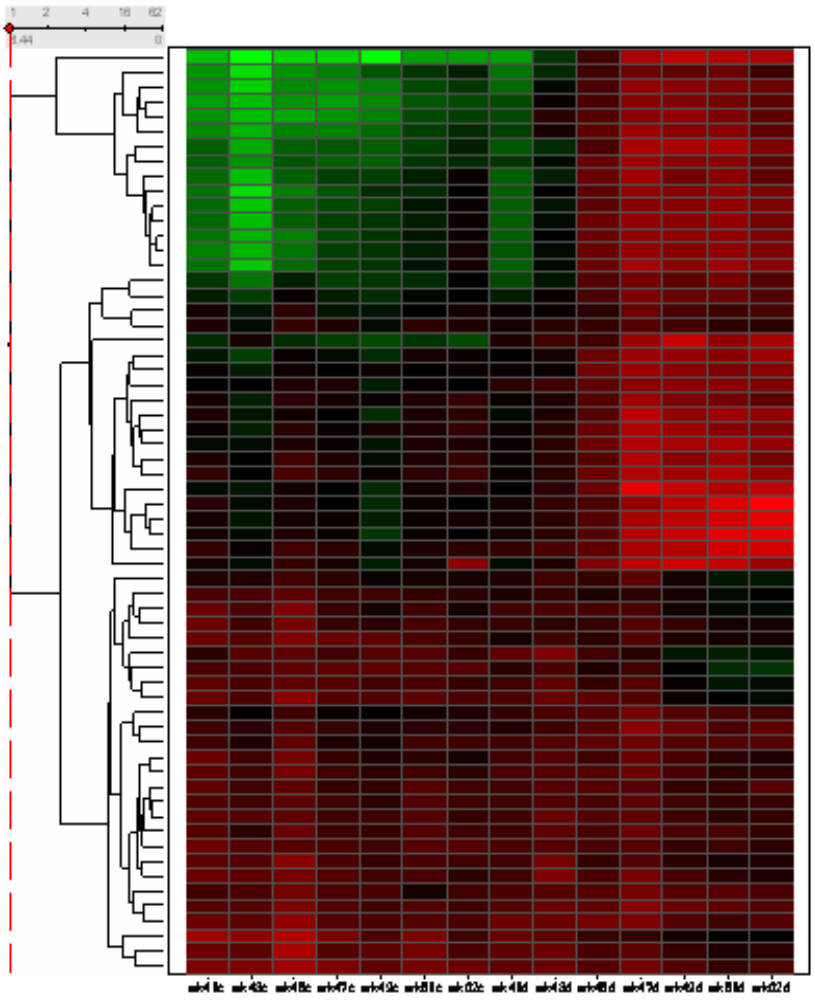


Figure 3.62 Hierarchical clustering of expression data from seedlings grown under controlled conditions. Column codes are week numbers combined with either a c (constant temperature and daylength) or a d (declining temperature and daylength). Hierarchical Clustering This visualization shows records in a dendrogram (a tree graph) based on the similarity between them. Calculation settings: Total no of records: 62 (All records); Columns included: wk43c, wk49c, wk47c, wk41d, wk51c, wk02c, wk41c, wk45c, wk43d, wk45d, wk02d, wk51d, wk49d, wk47d
Empty values replaced by: 0. Clustering method: UPGMA (unweighted average). Similarity measure: Euclidean distance. Ordering function: Average value

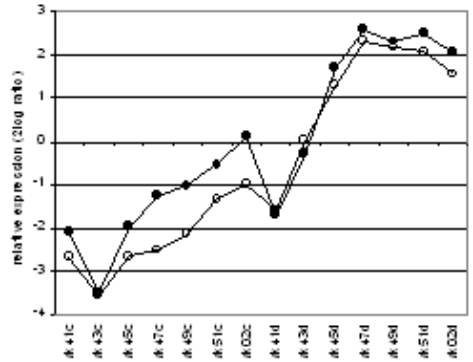


Fig.3.63A

Clone identity	Suggested function
open circles	
b1nr013	dehydrin
b1nr024	unknown
b1nr033	dehydrin
b5nr083	dehydrin-like protein
b6nr027	dehydrin 1 - Commerson's wild potato
closed circles	
b1nr021	unknown
b1nr035	unknown
b1nr038	unknown
b1nr039	unknown
b4nr049	unknown
b6nr008	unknown
b6nr067	unknown

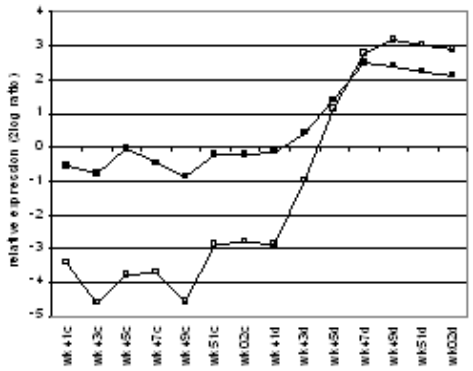


Fig.3.63B

Clone identity	Suggested function
open squares	
b1nr014	ABA-inducible protein
closed squares	
b1nr034	unknown
b1nr048	unknown
b5nr052	PRL1 associated protein - related
b5nr086	eukaryotic translation initiation factor 4A-1 (eIF4A-1)
b5nr087	unknown
b6nr056	unknown

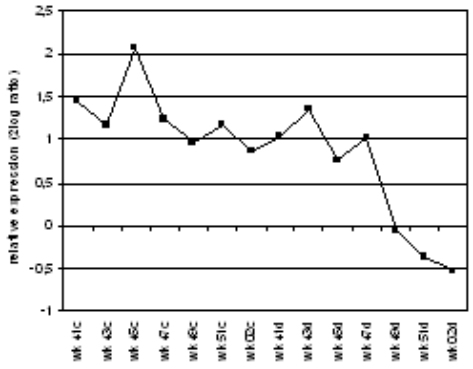


Fig.3.63C

Clone identity	Suggested function
closed squares	
b1nr005	GDSL-motif lipase/hydrolase protein
b1nr019	arabinogalactan protein
b1nr025	unknown
b1nr027	unknown
b1nr041	unknown
b1nr074	unknown
b1nr082	allergenic isoflavone reductase-like protein
b2nr041	alpha-tubulin
b2nr055	Sucrose synthase (Sucrose-UDP glucosyltransferase)
b2nr093	cellulose synthase catalytic subunit
b3nr031	Geen Omschrijving
b3nr056	expansin- related
b3nr066	GDSL-motif lipase/hydrolase protein
b3nr078	chalcone synthase
b3nr090	polyphenol oxidase
b4nr022	diphenol oxidase laccase

Figure 3.63 Different profiles derived from the Hierarchical clustering displayed in Figure 3.62.
A. Genes that show increased expression in the constant climate (c) as well as in the declining climate (d).
B. Genes that show stable expression in the constant climate and increased expression in the declining climate.
C. Genes that show stable expression in the constant climate and declining expression in the declining climate.

From week 33 (August) on, seedlings were put in a climate room where temperature and daylength were kept constant. Frost tolerance development was compared to a climate where temperature and daylength were changing (control situation), simulating field conditions. Freeze induced shoot electrolyte data are shown in Fig.3.61A. It is shown that under constant temperature/daylength a delayed development of frost tolerance took place from week 39 on. The delay is at certain points 4 weeks and, within the examined period, frost tolerance never reached the same level as in the control situation. For comparison SEL diff-15ºC (%) data of seedlings planted at the same location (Denmark) but in the field were included. Data from the climate room where temperature and daylength declined gradually were much alike these field data. Figure 3.61B shows that the seedlings planted in the field experienced lower temperatures slightly earlier than the ones in the climate room.
Dormancy profiles, measured as bud break potential, were comparable (Fig.3.61C). Hierarchical clustering (Euclidean distance) of the expression data from a selection (no missing values allowed) of the genes presented in Table 3.8 is shown in Fig.3.62. In general the expression of most genes hardly changed in the constant climate whereas the expression patterns for the genes found in the control situation were the same as found in the seedlings from the field. Examining the clusters reveals three characteristic expression profiles (Fig.3.63). Fig.3.63A shows a group of genes with increasing expression in the constant climate and a sharp rise in the control situation. Most dehydrins appeared to be part of this cluster next to a set of genes with unknown identity. Fig.3.63B shows a group of genes with stable expression in the constant climate and a sharp rise in the control situation. A clear difference in expression level at week 41/43 could also be observed for these genes. The group with the lowest level of expression consists solely of ABA-inducible protein homologues (open squares). Fig.3.63C shows a group of genes with constant expression in the constant climate and decreasing expression in the control situation. This is the largest group and within this group no genes could be observed with declining expression in the climate where temperature and daylength were kept constant.

3.9.3 Frost tolerance development in relation to differences in experienced cold
One series of apical buds from two-year-old seedlings was used for microarray hybridisations. In general, expression patterns of the genes were comparable with patterns found in the buds from one-year-old seedlings. Searching for genes that cluster together (Euclidean distance) with normalised frost tolerance (SEL diff-15ºC (%)) values revealed one dominant set of genes namely all ABA-inducible protein homologues (Fig.3.64). Genes that showed declining expression during the experimental period and that clustered together with the frost tolerance values were hard to find. And if so, by allowing larger deviations (Spotfire), they did not match the ones that were found for the one-year-old seedlings (Table 3.8).

3.10 Development of molecular diagnostic assays
Based on the expression patterns of two dehydrin genes a test system was developed for assessment of hardiness of pine seedlings. The test is based on the principle of RT-PCR (cDNA synthesis followed by amplification of specific gene fragments) combined with immunodetection of the amplified gene fragments. For immunodetection the Nucleic Acid Lateral Flow Immuno Assay (NALFIA) was used (van Amerongen and Koets, 2005). A mixture containing the amplified fragments is applied to the lateral flow system. At specific locations on the membrane antibodies are immobilized that capture specific labels attached to the amplified fragments. A second carbonated compound that binds to each amplified fragment is used to visualize the fragments on the membrane (Figure 3.65). Use of this assay system is simple, cheap and results are clear.
For performance of RT-PCR RNA has to be isolated from tree tissue. Preliminary data, obtained from pine trees of Dutch origin, suggests that needle tissue can be used equally well as bud tissue.
Figure 3.66 shows a typical result for one-year-old pine buds, provenance A70 planted at DIAS, harvested at two weeks intervals in 2001. The change in ratio of the signal intensity of both dehydrin genes indicates when the seedlings have become frost tolerant (from week 43 on).

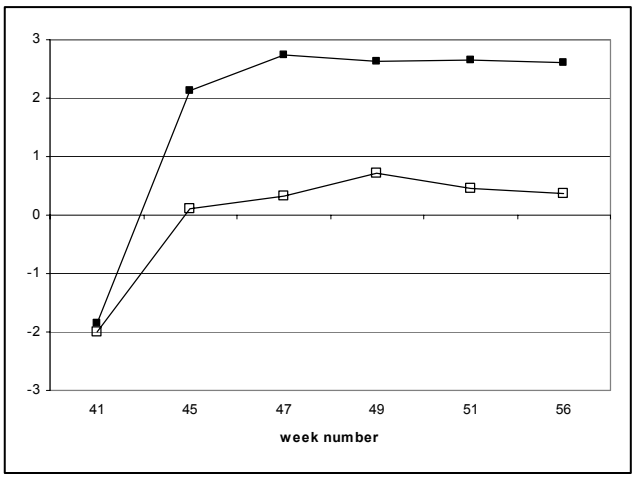


Figure 3.64 Comparison of frost tolerance development with expression of the ABA-inducible protein in two-year-old seedlings planted in Denmark, 2002.
Open symbols represent normalised SEL diff-15ºC (%) values. Closed symbols represent relative expression data from the microarray of the ABA-inducible protein.

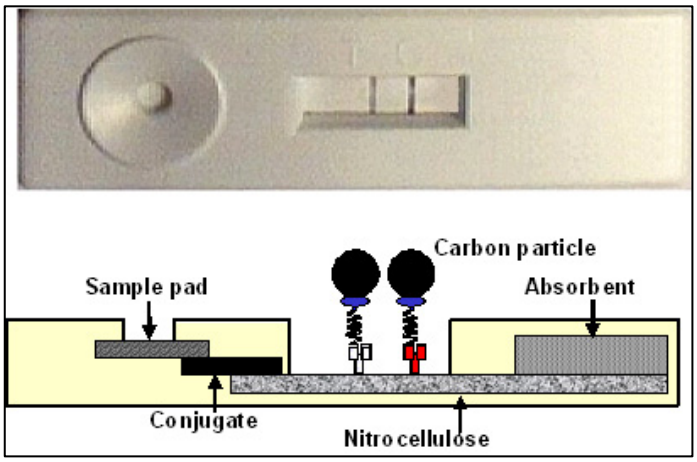


Figure 3.65. Principle of the NALFIA. In this example two lines are indicated whereas in the pine frost tolerance test a third line is added to the system.

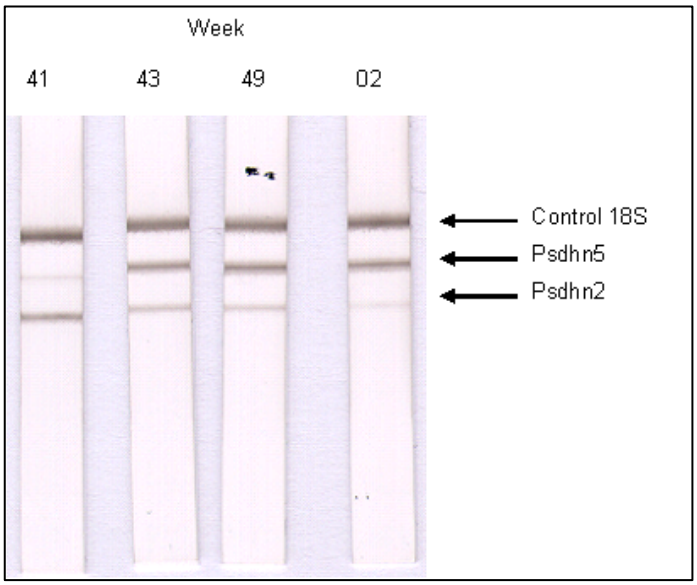


Figure 3.66. Test result of A70 apical buds. The seedlings are considered to be maximally frost tolerant from week 43 on.

3.11 Constructing an integrated database

In anticipation of the dbase, partners adopted a protocol to uniquely identify each sample. This made the integration of datasets relatively easy, as there was common key to act as a conduit between the various datasets.
The data from each partner was imported successfully and created a database of approx. 10 MB, containing more than 0.5 million entries. A data modelling process eliminated a lot of repeated data which would have further inflated the physical size and number of entries. De database harbours a large set of different parameters describing both physiological and molecular results and the experimental details needed for interpretation. There are around 22 physical, 35 PCR and 12 Microarray parameters. A user-friendly interface tool was built around Microsoft Access. This allows users to define their own queries against the integrated database of physiological and molecular data. For instance data concerning a specific provenance of tree species can be retrieved and the results constrained by temporal, physiological or molecular factors.

4. Discussion

4.1 Field experiments

Field trials were performed on beech and pine according to the same experimental protocol at the various locations. Next to a standard provenance, at each location various local provenances were grown. This allowed comparisons for most parameters that are known to influence dormancy and cold hardiness development such as geographic location (day length), genetic background and adaptation history. Pine and beech represent broadleaved and gymnosperm trees, but differ in dormancy profile as well. Beech develops much deeper dormancy than does pine.
The field experiments therefore resulted in data covering the variation in dormancy/hardiness encountered in practice to a large extent. Samples for molecular analyses, taken from these field grown trees, were eminently suited for studying the differential gene expression accompanying this biological variation.

4.1.1 Pinus sylvestris

High SELdiff values at the end of storage corresponded to high mortality of outplanted stock that had been established for two years. This is an indication of that the shoot electrolyte leakage (SEL) can be used to describe the seedling vitality. Seedlings with high survival (> 85%) had SEL values below 10%. This corroborates with REL estimates of bare-root trees of Douglas-fir, Japanese larch and Sitka spruce where high REL values corresponded to poor survival for cold-stored seedlings (McKay 1992). It therefore appears that under UK conditions an operational estimate for the lifting of pine seedlings is provided where SELdiff -15°C value <10%. For cold storage a safer margin is required, estimates can be drawn from the Danish study and operational lifting to cold storage (-2°C) of pine seedlings is considered when SELdiff -25°C value <10%. In this study the REL value could be used to describe seedling vitality though it is likely that the REL estimation technique is poorly adapted for investigations of containerised stock where cold storage is considered (see section 3.6). This is most probably as a result of containerised trees maintaining active root growth throughout the winter due to the elevated temperature retained within the peat plug in which they grow. Furthermore in the UK grown material storage was cool (+4°C) - a temperature which would be unlikely to induce hardening of the fine roots.
Temperature and photoperiod are considered to be the primary factors responsible of shoot acclimation and de-acclimation (e.g. Taulavouri et al. 1996). *P. sylvestris* of Scottish origin has been found to require greater chilling than Finnish or Russian origin plants (Leinonen 1996). In these trials later development of cold hardiness was evident in Sweden for A70 when compared to seedlings of native provenance (Figures 3.4 and 3.5). Very similar patterns in cold hardiness development, in both roots and shoots, were obtained for Danish and UK grown A70 material (Figures 3.3, 3.4 and 3.5). However, it is apparent that the degree of cold hardiness attained under Danish conditions was greater then that observed in the UK in year 1 (see Figure 3.3 SELdiff -25°C). This is explained by reference to the environmental thermo periodic data, which shows that the temperature experienced in Denmark was more severe, whilst the daylength cues are identical. Similar patterns were evident in the subsequent two years of study (Figure 3.4 and 3.5). Within location provenance effects were significant only in Swedish grown material, which reflects the severity of the climate at this site and the ill-adapted nature of the standard provenance to the environmental cues at this location, which is the most distant from the site of origin for this provenance. This highlights the fact that both the physiological and molecular tools developed might be applied in screening of genotypes for their suitability to ‘new’ climates. Provenance transfer, within safe limits (i.e. with the aim of ensuring the avoidance of late spring frosts) is a major concern for tree breeding programmes (Canell 1984, Hanninen 1996).
Dormancy in field grown pine appeared to be in response to combined photoperiod and thermo period environmental cues in the UK, where initial development of dormancy is in response to reducing daylength and the second stage in response to low temperature as has previously been reported (Taulavuori et al. 1997) . Dormancy did not develop in the UK grown material in the first year. This is probably in some small part an artefact of the tender nature of seedling trees starting the trials in year 1, but most probably results from the low cold temperature accumulation experienced in this

year (see Figure 3.8). True dormancy development occurred in the second and third years of study, and this was a response to accumulated low temperature. It has previously been reported that age-age correlations for autumn phrenology is especially complex in the early few years (Aitken and Hannerz 2001). We observed free growth characteristics in year 1, which lead to underestimates of DBB as second growth occurred shortly after temporary bud set. Of particular interest is the observation that A70 material lost dormancy before the local native N401 seedlings. Whilst not surprising it is indicative of the 'plus tree' selection system that was imposed during selection of superior individuals for material, now retained within this seed orchard, has arrived at a group of individuals whose growing season is longer, by dint of their genetics requiring a lesser 'signal' for the loss of dormancy and recommencement of active growth. This is a direct result of selection for growth rate characteristics. The effect of age was also evident in that 2-year-old material obtained a lower level of cold hardiness. This finding has important bearing on advice regarding the interpretation of this physiological test, for the nursery industry. Dormancy release, in year 3, was earlier in A70 though this was again, in part, the free growth character of A70 1-year-old material.
The driving environmental cues for the development of cold hardiness are evidenced in Figure 3.10. It appears that the primary cue is that of daylength but the modifying influence of temperature is also apparent. Specifically the day/night thermal difference appears, on this initial analysis, to be the key thermo periodic cue. This finding has not yet been reported elsewhere. This information could be utilised, through the re-analysis of existing datasets, for the testing of hardiness and budburst models, which may then improve attempts at modelling such behaviour (cf. Hänninen et al 2001).

4.1.2 Fagus sylvatica
High SELdiff values at the end of storage corresponded to high mortality (or poor growth) of outplanted stock. This is an indication of that the shoot electrolyte leakage (SEL) can be used to describe the seedling vitality. Seedlings with high survival (> 85%) had SEL values below 10%. It therefore appears that under UK and Danish conditions an operational estimate for the lifting of pine seedlings is provided where SELdiff -15°C value <10%. For cold storage a safer margin is required, estimates can be drawn from the Danish study and operational lifting to cold storage (-2°C) of beech seedlings is considered when SELdiff -25°C value <10%.
Temperature and photoperiod are considered to be the primary factors responsible of shoot acclimation and de-acclimation (Taulavouri et al. 1996; Heide 2003). *Fagus sylvatica* has previously been found to require chilling in combination with long daylength for the release of winter dormancy (Heide 1993b). In these trials release from dormancy occurred with increased exposure to low (ambient) temperatures. Release from maximum dormancy has previously been reported for broadleaved species and followed patterns similar to those observed in this study (cf. Heide 2003). This is likely to counteract the elevated spring temperature effects commonly reported of earlier flushing. However, the onset (early stages) of dormancy induction has not yet been reported. Whilst similar patterns were observed between two countries (Denmark and UK) of very similar latitude, thereby 'negating' the effects of daylength, in the first year of study, subsequent year's assessments were significantly different between locations (Figure 3.14, 3.15, 3.16). Specifically high levels of bud dormancy were evident from the start of trials over the second two years of study in the UK. Natural environmental chilling prior to testing was lower, at this site, in this early phase. Therefore we suggest that assessments of bud dormancy, as evidenced by the days to bud break test, are an inappropriate measure of plant physiological state.
Very similar patterns in cold hardiness development, in shoots, were obtained for Danish, UK and Dutch grown material. However, it is apparent that the degree of cold hardiness attained under Danish and Dutch conditions was greater than that observed in the. This is explained by reference to the environmental thermo periodic data, which shows that the temperature experienced in these countries was more severe, whilst the daylength cues are very similar. The UK experiences a more maritime climate and as such any provenance transfers, from other European countries, should take into consideration the potential effect on nursery operations and the 'ecological fitness' of such non-native stock. Similar patterns were evident in the subsequent two years of

study (Figure 3.16). This highlights the fact that physiological and molecular tools might be applied in screening of genotypes for their suitability to 'new' climates. Provenance transfer, within safe limits (i.e. with the aim of ensuring the avoidance of late spring frosts) is a major concern for tree breeding programmes (Canell 1984, Hanninen 1996), and our findings suggest that a further concern (and investigations) regarding their ability to develop full dormancy and cold tolerance are warranted. It has previously been reported that age-age correlation for autumn phenology are especially complex in the early few years (Aitken and Hannerz 2001). In two-year-old material of the non-local beech provenance earlier development of cold hardiness was evident. This reflects both the acclimation of these seedlings and suggests that for normal nursery operations where utilisation of novel or 'alien' origin material is considered exposure to local environment (over two growing seasons) may go some way to avoiding the potential of large scale crop failure at outplanting. A minimum requirement for long distance provenance transfer or the selection of new species, as is often advocated under climate change scenarios, should initial testing of the overwintering capabilities of selected stock under local environmental cues. To this end the suite of physiological and molecular techniques developed during Cold Tree provide a first step in the assessment of successful transfer of forest material.

4.2 Climate room experiments

The experiments in controlled environment were aimed at separating dormancy and cold hardiness development as much as possible. This did not fully succeed. The two phenomena are even more entwined than we expected. Another aim was to dissect the effect of the environmental triggers influencing the preparation for winter conditions. A problem encountered here was the fact that onset of events was pre-programmed. Both in beech and pine hardiness/dormancy development was initiated in total absence of the known triggers. There are two possible explanations for this phenomenon: either the main trigger is located before the examined period or the annual rhythm is genetically determined and will even develop in seedlings grown in constant climate from germination on. Further research is required to select between these options.

4.2.1 Pinus sylvestris

From physiological measures it appears that, for pine (and also for beech), the biological processes related to protection against winter conditions are pre-programmed to a much larger extent than we assumed beforehand. This idea is reinforced by the fact that, just as in the previous season, plants in the constant control climates still developed a certain level of dormancy and hardiness. Furthermore shoot based assessments did not show significant effects of SCOLD and SWARM treatments suggesting that the inherent biological rhythms are at least in some part able to overcome the 'confusion' of non-natural environmental conditions

The lack of any clear picture for the root-based assessment indicates that plants did not develop root hardiness in response to the controlled environment conditions. This is not unexpected for roots which are already cold tolerant but only semi-dormant.

Under controlled conditions declining temperature and daylength did lead to a decrease in dormancy levels, which was not evident for control climate treatment material (Figure 3.20). Cold hardiness development also more closely mimicked results obtained from field-based trials. This suggests a 5°C-day/night-temperature difference is 'critical' in dormancy development for this species (and provenance). This is in accordance with Dormling´s studies on Scots pine (1986). A truly dormant seedling does not grow even if the climatic conditions are favourable. These 'declining' treatment UK 1-year-old seedlings however showed a decline in the number of days to bud burst from week 41 to 51, which provides evidence that these seedlings, though not truly dormant, were less responsive to the growth promoting conditions in early autumn.

The fact that the initial rate of cold acclimation (week 39 - 45) was the same over both controlled environment declining conditions (Figure 3.18) and ambient (field grown) environments suggests that a common mechanism is active in these seedlings, and that the triggers are a combination of both daylength decline and accumulated cold sum. Some plants can regulate their growth through winter by only growing when the conditions are suitable. They are said to undergo imposed dormancy when there is no

distinct resting phase (as is present in true dormancy). This appears to be the type of response evident in the first year of controlled environment trials.
Declining daylength is an important environmental trigger for the onset of dormancy. Because daylength is consistent from year to year, it is a more reliable signal than temperature, which can fluctuate causing abnormal responses in plants. The growth regulator abscisic acid (ABA) is produced in increasing amounts in response to increasing night duration, promoting the development of dormancy through autumn and winter (Clapham et al. 2001). Indeed one of the putative markers identified in Cold Tree is an ABA responsive protein.
Temperature, although not thought to be as important as daylength, also plays a part in the onset of dormancy: whilst low temperatures alone are not enough to cause dormancy, in combination with short daylength, they can speed up its development. Although it is short days that cause a plant to enter pre-dormancy, low temperatures are believed to be need for true dormancy to occur (Taulavuori et al. 1997). To be released from true dormancy, plants need to accumulate hours at low temperatures and this explains why declines were evident in Year 2 climate manipulations.
The geographic origin of plants has also been researched in relation to a response to daylength, 1993a). The results vary between species: latitudinal and altitudinal effects have both been shown to affect the critical daylength for growth cessation and dormancy induction in Norway Spruce (Heide 1974).
Plants are protected from the risk of frost damage in 'winter' due to an increased length of warm temperatures (thermal time) being required for a plant to break dormancy after a reduced chilling season (Cannell & Smith 1986). Previously Murray et al (1989) investigated the impact climatic warming might have on the date of budburst in a number of tree species in Britain, finding that only species with low chilling (days <5°C) and thermal time (days >°C) requirements flushed earlier as a result of climatic warming. In our studies it is evident that day/night thermal difference is crucial to the development of 'true' dormancy in Scots pine seedlings.

Many models have been formulated to try and predict possible outcomes of global warming on the timing of budburst in a variety of species from a variety of locations. These models reproduce varying effects of climatic warming on budburst and the resulting potential for frost damage to plants. Published climate warming studies on *P. sylvestris* (Hanninen 1995, Leinonen et al. 1997) show that advanced date of budburst as a result of climatic warming and the possible risk of frost damage is less than that predicted by models. This study suggests that such predictions may benefit the remodelling of frost damage in the light of the thermal day/night difference shown here.
In response to the development of cold tolerance noted in the declining temperature and daylength climate room environment in Year 2, analysis is ongoing to elucidate the key drivers for onset of dormancy and cold tolerance in 'field grown' pine seedlings. Initial analysis, for the UK material, suggests that the 'trigger' for development of hardiness is indeed in part a response to the cumulative number of day/night 5°C difference. Full analysis of the general applicability of this 'signal' for pine, is pending collation and reinterpretation of the raw environmental data from the Denmark and Sweden experimental sites, which should provide supporting evidence for this hypothesis. The necessary data has been collected at the three North European sites involved in this project.

4.2.2 Fagus sylvatica
From the physiological measures employed it appears that, for first-year beech seedlings, the biological processes related to the development of protection against winter conditions are pre-programmed to a larger extent than previously envisaged. This idea is reinforced by the fact that plants under constant control climates continued to develop a certain level of dormancy and cold hardiness, in both years of study. Furthermore shoot based assessments did not show significant effects of SCOLD and SWARM treatments suggesting that the inherent biological rhythms are at least in some part able to overcome the 'confusion' of imposed non-natural environmental conditions of shifting temperature. The lack of any clear picture for the root-based assessment (data not shown) indicates that plants did not develop root hardiness to the level tested (-5°C) in response to the controlled environment conditions. In a sister

study field-grown containerised seedlings of beech were not observed to develop root cold hardiness to –2°C (section 3.2). This result is not unexpected for roots, which have the ability to develop some cold tolerance but are only semi-dormant i.e. they can grow under favourable temperature conditions. The main environmental factor controlling cessation of root growth and cold hardiness development is decreasing soil temperatures (e.g. Weiser, 1970; Ryyppö et al. 1998) which are buffered in containerised growth systems, and in these trials beech root would not have experienced temperatures below 0°C.
On the other hand, the results shows that parameters other than the well documented influences of temperature and daylength, may contribute to development of shoot frost tolerance (SFT), as observed in control climate manipulations. The fact that the initial rate of acclimation (week 37 - 45) was the same over a range different artificially imposed environmental conditions adds weight to the premise behind the Cold Tree project: namely that a common set of molecular markers should be evident for seedlings in the same physiological state. In fact a few published studies have pointed towards the potential of both dehydrins and ABA proteins in cold tolerance development (Rinne et al. 1997; Welling et al. 1997).
Variation in timing of bud burst within the test population (provenance) was low, i.e. individuals within a population behave in the same way. This would be expected if there is a strong stabilising selection for date of bud burst: if buds burst too early trees are susceptible to frost damage, if they burst too late they have a reduced growing season and growth rate. Thus natural selection ensures that bud burst occurs in a narrow window of time and there is little genetic variation for it within a population, although considerable variation is likely to exist between populations as a result of adaptive genetic variation to different climatic conditions at the site of origin.
In September/October buds of *Fagus* are still in a state of pre-dormancy and only enter a fully dormant state in November i.e. week 45 (cf. Vegis, 1964). In this study it appears that beech seedlings from all controlled environment trials entered almost equal levels of dormancy (number of days to terminal bud break under favourable growth conditions). In the first year of trials declining temperature (climate 3) seedlings, for a relatively short period, seemed to be more dormant (weeks 45-47). The intercomparison of results of field and climate room trials allows us to separate the effects of different environments and seedling age on the physiological development of cold tolerance and dormancy in beech. The declining conditions (Year 2) quite closely simulated the climatic conditions observed in the nursery, with respect to average temperature and daylength. The major difference between controlled and ambient climates were the shifts between day and night temperature in the nursery (around 5°C) and in the climate rooms (2°C) and, hence, the earlier development of SFT in the nursery. Whilst dormancy is normally ascribed to induction by shorter day lengths it does not appear independent of temperature from this series of investigations. Seedlings in both climate regimes (Year 2) entered a dormant state beginning in week 43 (constant) and in week 45 (declining). A positive effect of high temperatures on dormancy development has previously been reported in Norway maple (*Acer platanoides* L.) (Westergaard and Erikson 1997). Seedlings experiencing control conditions (climate 1) were observed to remain dormant later, while climate 2 seedlings started to release from dormancy from week 47. The combination of declining temperature and daylength, in year 2 trials, suggests that seedling beech may already have 'received' the necessary daylength cues for onset of bud dormancy, as evidenced from the increase in DBB noted for the control climate plants. However, the accumulation of cold, under declining conditions, led to an earlier onset of release of dormancy for the declining treatment plants. Low temperature (chilling) has previously been identified as the main environmental factor for loss and subsequent release of bud dormancy in temperate trees and shrubs (Vegis 1964).

4.3 Storability assays

High SEL values at the end of storage corresponded to high mortality. This is an indication that the shoot electrolyte leakage (SEL) can be used to describe the seedling vitality. Seedlings with high survival (> 85%) had all, except one treatment, SEL values below 13%. McKay (1992) showed in a study on bare-rooted Douglas-fir, Japanese larch and Sitka spruce that high REL values corresponded to poor survival for stored

seedlings. In this study the REL value could not be used to describe seedling vitality. The reason to this can be that the electrolytes from damaged seedlings are washed away while rinsing peat from the roots as preparation for the REL estimation.

It has earlier been shown that seedlings that are put into frozen storage in autumn initially increase in root freezing tolerance and later on, in late winter/early spring, decrease in root freezing tolerance (Lindström & Stattin, 1994). In this study, however only the shoots showed a dehardening in the storage. Temperature and photoperiod has been considered to be the factors responsible of shoot de-acclimation (see e.g. Taulavouri et al. 1996) The decrease in shoot freezing tolerance in the climatically stable environment in the frozen storage in this study indicates that more complex regulation may be involved in the dehardening.

The result shows that there is a risk to overestimate the seedlings storability if the decision solely is based on the shoot freezing tolerance. The root is the most sensitive part of the seedling to freezing and the temperature in frozen storage may severely damage the root system. Since the freezing tolerance of roots mainly is regulated by temperature it is important, especially early in season when days still may be warm, to check the root freezing tolerance.

4.4 Provenance effect

4.6.1 Fagus sylvatica

The bare-rooted beech seedlings of all provenances and ages used in this study all developed shoot freezing tolerance as could be seen from a decline in SELdiff-25°C and EC during autumn. No significant differences between provenances were observed. The development of frost tolerance was slower in 1-year-old seedlings compared with 2- or 3-year-old seedlings. This could be concluded from SELdiff-25°C measurements in two subsequent years and from EC measurements in the first year. In the second year, in which the decline of EC was similar for all ages, a different method for measuring EC was used than in the first year. Like in beech SELdiff-25°C measurements in Scots pine seedlings also indicated that the development of frost tolerance in 1-year-old seedlings was slower than that in 2- or 3-year-old seedlings. The results of SEL and EC from beech seedlings collected at commercial nurseries were in agreement with those of the field trials in this study.

4.6.2 Pinus sylvestris

For the pine seedlings there was a slight decrease in root electrolyte leakage (baseline) in early autumn. This is normal and reflects that the roots become more lignified as root growth decreases and the roots grow older.

The difference in root freezing tolerance between the 1-year-old and the 2- & 3-year-old pine seedlings can be a true age effect but it can also be an effect of the root sampling. I.e. the root system of an older seedling consists to a greater degree of lignified roots that are more freezing tolerant. This change in root constitution makes the older seedling more tolerant to freezing (Lindström & Mattsson, 1989). However, if the root samples in this study from the seedlings of different ages were of the same kind of roots the results actually show an age effect. An effect that makes the newly formed roots of 2- and 3-year old seedlings become freezing tolerant earlier than the newly formed roots on the 1-year-old seedling. However, this can not be verified in this study since root characteristics were not analysed in this study.

The difference in shoot and root freezing tolerance between provenances that was found in this study might be even greater than the results show. It must be considered that approximately two thirds of the 2- and 3-year-old A70 seedlings died in the winter 2002/03. The surviving seedlings were scattered in the block and not confined to special areas (edges – frost risk, patches – moulds). This indicates that the “hardiest” seedlings survived and that the tests made in 2003 were made on the more hardy individuals of the provenance. The results will therefore not reflect an average A70 seedling grown in Sweden.

Apart from the fact that the A70 seedlings were later in developing freezing tolerance and from the fact that they did not achieve the same level of freezing tolerance as the

Swedish provenances, there was also a tendency that the A70 seedlings lost shoot freezing tolerance in late autumn/early winter. Since determination of the freezing tolerance stopped early winter when snow covered the seedlings in week 48 this could not be confirmed in this study.

The standard deviations of the SELdiff and RELdiff values were generally high in this study. This implies that more seedlings should be used when testing the freezing tolerance with this method.

The provenances Bogrundet and Våge (commercial seedlings) have almost the same geographical origin. The difference in the development of shoot and root freezing tolerance between these provenances depends on seedling treatment in the nurseries but also on the nurseries geographical location and local climate. The Våge seedlings were raised at almost 5 latitude degrees south of their geographical origin. The photoperiodic signal for bud set, i.e. long nights, is given at an earlier date in the nursery Nässja than in Bogrundet. This is probably the main reason for the shoot freezing tolerance developing earlier in Våge seedlings compared to Bogrundet seedlings. Root freezing tolerance is known to be mainly controlled by soil temperature but is also influenced by the development of the shoot freezing tolerance (see Smit-Spinks et al, 1985). However, soil temperature data to support this were not collected in the commercial nurseries.

The impact of photoperiod on growth was also shown at the start of the bud break tests in the difference between numbers of seedlings that had formed buds. Bud set is regulated by night length and seedlings of A70 provenance, a provenance that is not adapted to the photoperiod in Garpenberg, had not responded to the night length in Garpenberg with bud set. Åmsele seedlings however, seedlings of the most northern provenance, were triggered to set bud quite early in the season when grown in Garpenberg.

The 1-year-old standard seedlings, irrespective of provenance, never showed a true dormant stage. This is in accordance with Dormling´s studies on Scots pine (1986). A truly dormant seedling does not grow even if the climatic conditions are favourable. The Swedish 1-year-old standard seedlings however showed a decline in the number of days to bud burst from week 36 to 44. This shows that these seedlings, though not truly dormant were less responsive to the growth promoting conditions in early autumn. The commercially grown Våge seedlings however, though only 1-year-old, displayed deep dormancy. Twelve of the 15 seedlings did not break bud within 90 days in growth promoting climate. These seedlings had been long night treated and were sown earlier than the seedlings of the standard provenance Åmsele that did not display such a deep rest. The small difference in age and the long night treatment might have affected the ability to develop dormancy.

When comparing the pattern of development of the seedlings freezing tolerance with the pattern of the development of rest/dormancy in the seedlings it is obvious that the state of rest/dormancy precedes the achievement of full frost tolerance. This is in accordance with earlier studies (see e.g. Colombo, 1990).

4.5 Transcriptional profiling

The general approach taken in transcriptional profiling of both beech and pine was aimed towards finding the major shifts in gene expression accompanying generally occurring physiological changes. For both species a dedicated microarray was used carrying pre-selected (by subtraction) genes. The beech array was smaller, because it was possible to make use of results from analyses in pine. For both species the field sample series were excellent material to reconstruct the general order of molecular events. Comparison between different provenances and geographic locations allowed distinguishing between differential expression related to the physiological developments of interest and noise effects induced by local environment.

Subsequent analysis of climate room grown samples allowed further correlation of the gene expression events to physiological developments.

4.5.1 Pinus sylvestris

A combination of climate room and field experiments was used to correlate gene expression to physiological characteristics and climatic conditions. Seedlings from different origins were subjected to different climatic conditions, and physiological parameters were determined. A microarray containing cDNAs, representing approximately 1000 unique pine genes was used to measure gene expression in apical buds from seedlings from these experiments, in order to directly compare physiological behaviour and gene expression profiles.

In the climate room experiment the influence of photoperiod and temperature on the development of cold hardiness was demonstrated by the differences in shoot cold hardiness (frost tolerance) found in the different climatic regimes (Fig 3.49C). Initially seedlings placed at all three climatic conditions improved their frost tolerance, but from week 41 on, the seedlings grown under warm, long day conditions (CR constant) did not further improve their cold tolerance. In a climate room with declining temperature and photoperiod (CR decreasing), shoot frost tolerance continued to increase over time, while control seedlings that were placed outdoors developed maximal levels of frost hardiness. Both shortening photoperiod and decreasing temperatures are effective signals for frost hardening of pine, and the responses to photoperiod and temperature interact (Beck et al, 2004; Zhang et al, 2004). In experiments where pine seedlings were exposed to different light regimes under ambient temperatures, short photoperiod (9 hrs) induced hardening already in late summer, while seedlings subjected to a long photoperiod (16 hrs) showed delayed hardening, which started only after the first exposure to subzero temperatures (Beck et al., 2004). This is in line with the hypothesis that short day conditions contribute to the development of hardening competence, and renders the seedlings more sensitive to the inductive effect of temperature (Beck et al., 2004; Zhang et al., 2004). In a previous climate room experiment during this project (data not shown), the effect of photoperiod and temperature was independently assessed, but no significant levels of frost tolerance induction were found. It is possible that the temperature and light conditions were sub-optimal for frost tolerance induction when applied independently. In that experiment a smaller difference between day and night temperatures was used (2°C), while the light levels in the cabinet (max light levels PPFD were approximately 350 µmol m^2 s^{-2}) were relatively low. When similar temperature and photoperiod regimes were applied in combination (decreasing day length with a larger day/night difference, and decreasing temperature, (CR decreasing, this study), Fig 3.49), the combined signal was sufficient to trigger moderate levels of frost tolerance in the shoot (Fig 3.49C), but not the root (Table 3.5). This could be due to the fact that root frost tolerance requires lower temperatures for induction (Zhang et al., 2004).

The physiological differences observed among the climate room treatments were used to identify a descriptive gene set, which was used to molecularly characterise the samples from the experiments, and allowed direct comparison of gene expression profiles to physiological parameters and climatic conditions.

In an experiment aimed at comparing the physiological and molecular response of the standard provenance (A70) to climatic conditions at three different locations, differences in onset and timing of cold tolerance induction were found, which seemed to parallel differences in day length and temperature. Seedlings exposed to the Swedish climate started increasing their cold tolerance first, but seemed to lose their frost tolerance prematurely as well, suggesting they were unable to cope with the harsh conditions, possibly due to its adaptation to the Scottish climate (Fig 3.52). Although the expression profiles of the descriptive gene set did not reveal major differences in timing of expression programs, expression of individual marker genes correlated well with physiological and climatic parameters (Table 3.6).

Finally, in order to assess the influence of genetic origin of different provenances on their physiological behaviour, the standard provenance A70 (from Scotland) was compared to local provenances in Denmark (Lindås) and Sweden (Åmsele). Here, clear differences between the two provenances were found at both locations, where the local provenance reached high levels of cold tolerance before the standard

provenance. This corresponded to the gene expression profiles, which indicated that local provenances had more “cold tolerant” expression profiled than the standard provenance. It has been documented that genetic origin plays a large role in the sensitivity to photoperiod, and that bud set, growth cessation and frost hardening occur earlier in northern provenances than in provenances from a more southern origin (Hurme et al., 1997; Repo et al., 2000).

Candidate marker genes for physiological characterisation
From the descriptive gene set, a limited number of genes were selected for a more close analysis of their suitability as molecular marker for frost tolerance. Genes that showed a large relative change in RNA levels over the season, and that had a good positive or negative Pearson correlation to SERdiff-25 were selected (Table 3.4). For most genes in Table 3.4, a putative function could be assigned, and the most likely homolog from Arabidopsis could be identified. For some genes in Table 3.4, no match could be found in the database at all, while for others the only match was an EST sequence from loblolly pine (*Pinus taeda*).
The genes that that were positively correlated to cold hardiness included antifreeze genes, late embryogenesis abundant (LEA) –like genes, metabolic genes and other stress or ABA induced genes.
Antifreeze proteins have been isolated from different plant species, and many were found to be homologous to pathogenesis-related (PR) proteins. PR proteins are normally induced by pathogen infection as part of the plant’s defence, and often have fungal cell wall degrading activity or can enzymatically and inhibit fungal enzymes. In addition to their antifungal properties, certain PR proteins were found to have antifreeze activity, since they were able to bind to the surface of ice crystals and prevent crystal growth. Such antifreeze-PR proteins include β-1,3-glucanases, chitinases, and thaumatin-like proteins (Griffith and Yaish, 2004). The class IV chitinase, beta-1-3 glucanase and thaumatin like genes that are expressed during cold acclimation in our experiments (Tables 3.4 -3.7) could have antifreeze properties as well, explaining their cold induced expression profile.
LEA proteins were first discovered based on their abundant expression in maturing seeds (Galau et al., 1986), and a number LEA proteins were shown to be induced by dehydration and cold and due to their hydrophyllic nature they are thought to act as molecular chaperones under water stress conditions, preventing protein aggregation during desiccation and freezing (Goyal et al., 2005). LEA proteins can be grouped based on conserved sequence domains, and members from different groups are thought to play different roles during desiccation and cold stress (Wise and Tunnacliffe, 2004). Dehydrins belong to the group 2 LEA proteins, and their expression and role during dehydration and cold tolerance has been studied extensively in a number of plant species (see review: Allagulova et al. 2003). We have found a number of LEA genes and dehydrins which are upregulated during cold acclimation (Tables 3.4 – 3.7), and could be useful as molecular markers for cold tolerance. Interestingly, not all dehydrins RNA levels increased during cold acclimation. Dehydrin 2 expression was not correlated to physiological parameters at all, while dehydrins 1 showed an inverse correlation. It is possible that different family members play different roles during the cold acclimation process, explaining their differential expression. These different expression profiles were exploited in the development of a molecular diagnostic tool, where the ratio between dehydrins 1 and dehydrin 5 was used as a measure for cold tolerance (data not shown, see exploitation and dissemination.
Metabolic genes that were induced during cold acclimation include a purple acid phosphatase (PAP), an epoxide hydrolase, sucrose synthase and malate dehydrogenase. Purple acid phosphatases hydrolyse phosphate esters, and are often induced under low phosphate conditions. The Arabidopsis genome contains 29 PAPs (Li et al., 2002). A NaCl induced soybean PAP has been implicated in the adaptation to salt stress, through possible scavenging of reactive oxygen species, a role the pine gene identified in our study gene might play during cold stress (Liao et al., 2003). Epoxide hydrolase is an enzyme in the methionin biosynthesis pathway. Metabolites such as disaccharides, proline and other sugars or amino acids may allow the trees to cope with the freezing conditions by increasing the osmolarity of the crucial bud

tissues. Both sucrose synthase and epoxide hydrolase are upregulated during cold acclimation.
Other stress or defence related proteins that were expressed during cold acclimation included pathogenesis related (PR) proteins, defensin, metallothionein, pinosylvin synthase and an aluminum induced protein. PR proteins are induced in many plant species by pathogenic attack or environmental stress. The two PR genes found in our study are related to PR10, which in many species is encoded by a large gene family. For example, in *Pinus monticola* a large PR10 family was identified, which members were differentially expressed in response to cold and wounding (Liu et al., 2004). Another gene that was expressed during cold acclimation was a defensin gene, containing a gamma-thionin domain. Plant defensins inhibit the growth of a broad range of fungi and are also induced upon pathogen attack (reviewed by Thomma et al., 2002). It is possible that these defense-related genes are expressed during cold acclimation to prevent fungal attack during the quiescent period in winter, when very little metabolic activity or de novo gene expression occurs. Metallothioneins are heavy-metal binding proteins, which have been reported to be expressed in a large variety of plant tissues, and induced by a number of different environmental conditions (Cobbett and Goldsbrough). The metallothionein found in our experiments appears to be a cold acclimation induced member of this large gene family. Pinosylvin is a stilbene phytoalexin which normally accumulates in heartwood of pine trees. Pinosylvin synthase is induced by various stresses but is not expressed in non-stressed seedlings (Preisig-Muller et al., 1999). Our results show that pinosylvin synthase is also induced by cold in pine buds. No putative function is known for the aluminum induced protein found to be upregulated during cold acclimation. In a study on drought stress in loblolly pine, this same gene was included, but one clone was upregulated while another was downregulated during drought stress (Watkinson et al., 2003).
Among the most rapid and highly induced genes was that encoding ELIP1 (Early Light-Induced Protein 1). ELIP1, as well as ELIP2, are nuclear-encoded thylakoid membrane proteins that are expressed in response to light stress, that are thought to be photoprotective pigment carriers or chlorophyll exchange proteins. Expression of this gene was also found to be cold induced in *Arabidopsis* (Fowler and Thomashow 2002), but its role in the cold acclimation process remains unknown.
Another group of highly expressed genes during cold acclimation was represented by 4 contigs with homology to a water deficit stress and ABA induced gene family from loblolly pine (Padmanabhan et al., 1997), encoding 4 putative *Pinus sylvestris* members of this gene family. These genes contained an asr (ABA stress and ripening induced) signature, which has been found in genes from many different plant species. Spots corresponding to all 4 contigs had similar expression patterns, which could be the result of cross-hybridisation among the four contigs. Finally, two transcription factors that might play a role in the cold acclimation process were identified in our experiments. These were a HAP3 domain containing CAAT binding factor subunit and a member of the E2F transcription factor family. The CAAT binding factor subunit was shown to be transiently downregulated by low temperature (8 hours after transfer to 4°C) in *Arabidopsis* (Fowler and Thomashow, 2002).
Genes that are down regulated during cold acclimation are also important potential candidate genes for cold tolerance, when used in combination with an up regulated gene. The relative amount of mRNA of the induced and down regulated gene allows an accurate prediction of the cold tolerance of the sample tested. A relatively small number of down regulated genes was found in this experiment, and most of these are represented by a single clone, reflecting the fact that the libraries used for the cDNA microarray were enriched for genes that were highly expressed in cold acclimated buds.
Many of the down regulated genes encode ribosomal proteins or ubiquitin, reflecting the decrease in protein synthesis and degradation activity during fall. The decrease in tubulin and xyloglucan endotransglycosylase mRNA levels during cold acclimation might reflect a drop in cell division and expansion, since the products of these genes are needed for cellular structure and cell wall elongation and restructuring respectively. Interestingly, 7 cDNA clones were found to encode calmodulin, which indicates its abundance in the cDNA libraries used to produce the microarray. Expression of this gene was negatively correlated to cold acclimation, and RNA levels showed a

moderate but reproducible drop during the cold acclimation process. Since Ca signalling is thought to play an important role during cold stress response, and different calmodulin family members have been shown to be transiently induced or repressed by cold stress (Fowler and Thomashow, 2002), the role of this gene in the cold acclimation process remains unclear.

4.5.2 Fagus sylvatica

In order to gain insight in molecular events taking place during hardening of beech seedlings, expression analysis of a set of relevant genes was performed using cDNA microarray technology. Selection of relevant genes was realised using cDNA subtraction technology at construction of the cDNA libraries. In this way we obtained a library enriched for genes active when the seedlings were proven to be frost sensitive and a library enriched for genes active when the seedlings were proven to be frost tolerant. Next to that, knowledge gained from gene-expression analyses in pine proved to be helpful in making a final selection of genes to be put on the microarray. We decided to spot special genes of interest, with regard to test development in a later stage, multiple times. Whenever possible we made use of different contigs of the same gene.

We paid special attention to the group of LEA genes (Wise and Tunnacliffe 2004) known to be involved in all kind of cold/salinity/drought stresses (Seki et al. 2001). We also found several cDNA clones in our libraries that display high homology with transcription known to be involved in mediating responses to the mentioned stresses. Among those are members from the C/DRE binding factors (Kim et al. 2002), from the MYB binding factors and members from the WRKY family of transcription factors (Chen et al. 2002).

We also made sure to include genes generally associated with cell growth, such as tubulins and expansins (Breyne et al. 2002, Li et al. 2003).

Expression profiles of 196 cDNA clones that matched the quality criteria were analysed in a single provenance (Bregentved), planted at two different locations in the same year. Despite the fact that there was a difference in experienced cold between the two, frost tolerance developed in parallel (Figure 3.56). This suggests that in both situations the trigger for frost tolerance development was the same. Because of the fact that they were planted at almost the same latitude, this result can be an indication that daylength is an important factor regulating frost tolerance development. Compared to research done in birch, where various effects of daylength and temperature were studied in detail (Welling et al. 1997, Puhakainen et al. 2004) this is not unexpected. Under natural conditions, northern ecotypes that were more responsive to shortening photoperiod than southern ecotypes display a difference in timing of frost tolerance development (Li et al. 2003).

In contrast, dormancy profiles are not comparable, making selection of genes associated with these profiles impossible. For both situations however holds that bud break potential is low before full hardiness was achieved.

Figure 3.57B makes clear that the way in which the selection of clones to be put on the microarray was made was very effective. Almost every clone present on the microarray displayed differential expression. Because of the fact that there are many genes that display a big change in expression we put the criterion for genes with so-called hardly-changing-expression high (delta 2 log ratio < 2). If needed, one can still search for interesting expression patterns within this group. Regarding the aim of this project however we are inclined to focus on the genes with clearly changing expression.

Figure 3.58 suggests an almost biphasic overall pattern centred on week number 47. From that moment on the seedlings were measured to be maximal frost tolerant (Fig. 3.56A).

Genes upregulated during frost tolerance development.

As expected, most genes with an assigned function in cold/drought stress appeared to become highly expressed during the examined period. Expression stabilised or even declined as soon as maximal frost tolerance was reached. On top of this list (Table 3.8A) are the so-called ABA-inducible proteins homologues. Although there are put 6 different contigs on the microarray, mutual homology is high (around 90%). Because

this protein is present in such a large number of spots on the microarray, the expression profile has a solid basis. This is furthermore established by using real-time RT-PCR to verify the data (Fig.3.60). The nature and function of this protein is largely unknown. Also in Scots pine (this study) the cDNA, encoding this protein, is present in high amounts in libraries made from frost tolerant material. Therefore it is supposed to be an important frost tolerance related gene.
Early light induced genes are mostly studied in relation to light-stress effects on plants but from these studies it appeared also that they are associated with other stress conditions, for instance salt stress (Savenstrand et al. 2004).
The presence of so many genes assigned unknown, indicates that a lot of knowledge about the mechanisms that plants exploit in order to cope with stress has still to be build.
With regard to the dehydrins, well known to be involved in protection of plants against several stresses with a dehydrate component (Close 1997), all of the different cDNA clones that were put on the array displayed the same profile. Because of the high degree of homology between the clones and because some dehydrins fall into the class of hardly-changing-expression, it can not be excluded that there are isoforms that are differentially regulated. However with this microarray-hybridisation technique it is not possible to discriminate clearly between them.
Because of the limited amount of data points it is not possible to state whether there exist differences in timing of increase of expression between the three clusters.

Genes downregulated when maximal frost tolerance levels are reached.
From Table 3.8B it is clear that the period before maximal tolerance is reached is an active one, from a metabolic point of view. A lot of genes related to a diversity of processes fell into these clusters. This also includes the cold/drought stress related transcription factors.
The fact that all of the tubulin genes are in this cluster suggests that the recent idea that tubulins are involved in stress signalling can be valid (Wasteneys 2004). On the other hand, taken together with the finding that both expansin and a xyloglucan endotransglycosylase precursor, both active in cell wall loosening (Li et al. 2003), are in this cluster suggests that cell growth potential is at its lowest level from week 47 on.
For two genes belonging to this group, expression was verified using real-time RT-PCR. Differential expression could also be easily determined using this technique (Fig.3.60).

Integration with physiological data.
Data from frost tolerance measurements were normalised in order to search for gene expression patterns that matches the profile. With regard to the genes that belong to the category with increased expression, the result of this search was unequivocal. The ABA-inducible protein homologues matched perfect. Genes that belong to the clusters that display decreased expression showed a less clear result. However, three different genes (Table 3.8B) can be assigned that match the profile in both situations (Denmark and Scotland). Such genes are good candidates for the development of rapid frost-tolerance-assessment tests.
The difference in quality of matching between genes that belong to the cluster with decreased expression and the cluster with increased expression is further illustrated in Figure 3.59. In general, candidates that belong to the functional class of cold/drought related genes appeared to be simultaneously upregulated in Denmark as well as in Scotland, like frost tolerance development. This is in contrast with candidates from the class of genes whose expression declined. Timing differences can be observed for most genes in this class that are neither correlated with differences in experienced cold nor with dormancy profiles.
It appeared difficult to find genes that correlate with the experienced cold (Cold Index). This can be an indication that once frost tolerance development is triggered, actual temperature does not have big influence on the process.
Dormancy profiles were so different in both situations that it was impossible to find any correlation with gene expression that holds for both locations (Denmark and Scotland). What can be concluded from our data is that metabolic activity in general is not a good measure for dormancy assessment. It is clear that until week 47, when at both

locations bud break timing is relatively high, metabolic activity is high. This holds true even for the genes with functions associated with cell growth, such as tubulins and expansin.

Delayed frost tolerance development correlates with a delayed shift in expression of frost tolerance related genes.
Data presented in Figure 3.61 makes clear that in the climate where daylength and temperature were kept constant, frost tolerance development is impaired. Frost tolerance development in the climate where both daylength and temperature decreased (control situation) resembles the field situation be it that there is a delay. Compared to the Cold Index data one could argue that the difference in experienced cold causes this shift in frost tolerance development. But based on our findings, by comparing the two field trials (Denmark and Scotland, 2001), this conclusion is not valid. It is more likely that a complex of differences in environmental stimuli is causing this delay.
Because dormancy develops more or less normal in the constant climate this situation seems ideal to strengthen the correlation of specific genes with frost tolerance development.
The general picture is that genes that were found to be clearly regulated in the field situation (the genes described in Table 3.8) didn't change in the constant climate (Fig.3.62).
Zooming in on several different clusters (Fig.3.63) makes clear that various genes react differently on this climate. Whereas dehydrins and a group of unknown genes showed increased expression in this climate, the group of ABA-inducible protein homologues together with several other genes showed stable expression. When the level of expression is compared with that from the control situation (Fig.3.63A) it is clear that this is much lower.
Our data suggests that there are early signals, most likely daylength, before week 33 that are causing this increased expression of the group of dehydrins and unknowns. It can also be that the applied constant climate itself triggers the increased expression.
For specific dehydrins of *Betula pubescens* it is shown that photoperiod and temperature can have different effects on expression of the genes (Welling et al. 2004). Exposure to relatively low temperatures, as applied in the constant climate, can also trigger frost tolerance development.
Still, frost tolerance is impaired in our constant climate and this is also clearly illustrated by the genes that show stable and low expression (Fig.3.63B).
In combination with the data obtained from the field grown plants (Fig.3.58A) it is possible that indeed dehydrins and others are early indicators of frost tolerance development whereas others, such as the ABA-inducible protein homologues, are late indicators. Moreover the latter ones can be considered as possible indicators for maximal frost tolerance.
The fact that all genes that show decreased expression in the control climate remained constant in the constant climate indicates that none of them is correlated with early events with regard to frost tolerance development. This confirms our results obtained with the field grown plants. From a practical point of view such genes can be very helpful in defining maximal tolerance.

Age effects
By studying the expression profiles of one field trial of two-year-old beech seedlings we did not encounter remarkable differences with one-year-old seedlings. Expression profiles were much alike but time-shifts were observed. This holds true especially for the genes that show decreased expression when hardening proceeds. So this is an indication that the way in which the general metabolic processes (including the ones that are needed to prepare the seedlings for maximal tolerance) decline, is subject to variation. This is illustrated by the fact that it was impossible to find similar genes with expression profiles that match the frost tolerance data.
Actually this is the same trend as found by comparing the two field trials of the one-year-old seedlings but more extreme.
Genes with increasing expression behaved more consistent, which strengthens the idea that these gene-products are really involved in protecting the plants to harsh environmental conditions.

4.6 Database construction

The use of spreadsheets did highlight some disadvantages of using these as intermediate data stores. For instance special formatting could cause data not to be read during import, which was only noticed by careful checking of the data in the database or by exporting the data back to the spreadsheet for comparison. However on balance the use of spreadsheets was still positive because of simplicity and the fact the Cold Tree partners had well defined standards for their data.

Retrofitting full standards such as Minimum Information About a Microarray Experiment (MIAME) was not possible because Cold Tree work was well advanced when these standards finally became established. Therefore in Cold Tree a subset of MIAME data was recorded and stored in the database.

A fully functional downloadable access database with all Cold Tree data will be made available to all partners through web hosting. The existence of this tool will significantly assist in the detailed analysis that is required when handling such extensive datasets. This tool will become publicly available to the research community after the IPR agreement ends (2009). Prior to that date the website will be password protected.

5. Conclusions

In this report the results of a close collaboration between tree physiologists and molecular biologists, are described. The aim was to increase insight in seedling physiology from a practical point of view, directed towards the development of functional monitoring tools for nurseries. From the results described above it is clear that all the components needed to perform molecular tests in commercial nurseries indeed have become available during the COLDTREE project: good protocols, marker genes, physiological interpreters and easy assay formats. Different assay formats could be compared, resulting in a fast, reliable molecular assay for cold hardiness that serves as a proof of principle. A large and highly valuable database was created that can be used to select new indicators for other tests. The Cold Tree project contributed largely to the *Pinus sylvestris* molecular resources and has created the largest *Fagus sylvatica* resource available to date.

Comparison of both trees led to interesting conclusions. In both trees is was shown that even in constant environmental conditions with respect to daylength and temperature a certain level of cold hardiness still develops. That means that either, the trigger for onset of this development is situated in time before the moment that the seedlings went into the controlled environment, or the development of cold hardiness is genetically programmed to a large extent.
The comparison of field grown and climate room grown plants enabled us to differentiate between genes involved in the early- or pre-programmed part of hardiness development and genes that are more responsive to actual environmental conditions. Correlations with cold index figures learned that cold hardiness, as measured by shoot electrolyte leakage is more responsive to daylength than to cold triggers.
Both in pine and beech, many of the genes upregulated during hardiness development are well-known stress-responsive genes. An important gene family in this group are the dehydrins. Expression of dehydrins was studied in more detail. It was shown that while the absolute expression levels of some dehydrin family members strongly increase during fall, their inducibility sharply decreases. That is support for the idea that winter buds have to make sacrifices with respect to responsiveness, since metabolism is minimised, and cope with that by maintaining a constant high level of protective proteins in the cells. The day/night temperature difference appears on initial analysis, to be the best candidate for inclusion with daylength when modelling the development of dormancy and cold hardiness in both beech and pine.

The obtained data are very helpful in selection of a set of genes that can be used as indicators for either one stage: the hardening stage or the fully hardened stage. The behaviour of the cold/drought related genes appeared to be more consistent than the ones that are involved in various other metabolic processes. So by making the selection a larger proportion of the set must consist of genes from the latter class. This is the class that showed decreased expression as soon as maximal tolerance levels were reached. Both for beech and pine a selection was made of potential indicator genes, all highly differential in fall, all highly correlated to cold hardened status and all high absolute expressors.

From the field trials it is clear that age effects are evident in both beech and pine and should be taken into account for the correct interpretation of any test. In pine a clear provenance effect was shown as well. In beech this effect was not observed. The reason for this might be that the beech provenances all originated from a relatively small range of latitudes. Another explanation might be that in beech hardiness development is less responsive to environmental conditions. This in turn, may relate to the fact that beech shows true dormancy, whereas pine merely has a period of quiescence. This is a question that needs further research to be answered.

As a proof of concept two differentially regulated dehydrin gene family members, and one control gene were converted into a molecular diagnostic assay, of which the reading of result is highly simplified and comparable with a pregnancy test. This test proved to work very efficient, even in the hands of not molecularly skilled persons. This

is one of the formats that may be used for the implementation of all molecular results into assays.

Physiological assays appeared to be very useful as well. The shoot electrolyte leakage (difference) test can be used as an indicator of the development of cold hardiness in container grown Scots pine and beech seedlings, this information is the type which can be used operationally to aid decisions regarding the ability of plants to withstand cool (+2°C – 0°C) and cold (-2°C – 0°C) storage. The root electrolyte leakage is not an indicator of seedling vitality for containerized seedlings.

The construction of an integrated database added value by centralising the data in one location, as opposed to a number of spreadsheets with many constituent worksheets. The database allows easier querying and analysis across physiological, molecular, environmental and experimental parameters. This combination of data in an integrated dbase will lead to a profound insight in the molecular pathways involved in the onset and release of winter hardening in *Pinus sylvestris* and *Fagus sylvatica*. Information on the influence of climate, environment and provenance on the expression of the genes concerned will become available, and will contribute to the definition of a general hypothesis on the molecular events underlying the onset and development of dormancy and cold hardiness in woody species.
The results obtained in Cold Tree made it possible to make a start with such a hypothesis, but they are not sufficient to come to final conclusions. Another limiting factor is the fact that the integrated database has only recently come available and analyses could not be completed before the deadline for this report. However, some general processes can already be distinguished.
Both pine and beech respond similar to environmental triggers. It appears that the influence of day/night thermal difference is important, alongside photoperiod, for the development of cold tolerance and dormancy. Absence of environmental triggers does not result in absence of dormancy and hardiness development
The crucial trigger for dormancy/hardiness development either occurs before the studied period, (this may be the shift between increasing and decreasing daylength in summer) or is genetically determined. To sort this out additional experiments are needed
In general the expression of stress (especially drought) related genes increases strongly during initial phase of dormancy development, but their inducibility decreases. Genes related to general metabolism, DNA replication, cell cycle activity and protein assembly decrease in the same period.
The line of events is probably triggered by shortening of daylength in summer. This initiates a cascade of biological processes including membrane rigidification, cessation of active growth, decreased expression of genes related to cell division, primary and secondary metabolism and increased expression of subsets of stress related genes leading to accumulation of protective compounds in the cell. The result of the process is a moderately hardened seedling, with (for beech) dormant buds. While this development is progressing it can be enhanced by other environmental triggers such as cold or, more likely, day-night temperature differences. This instigates a secondary course of events leading to enhanced expression of other stress related genes, stabilisation of membranes and finally increased cold hardiness. In beech the cold trigger releases dormancy as well. However, further analysis is needed to assign specific genes to this event.
From the presented results it is clear that the stage before maximal frost tolerance levels are reached is a metabolic active state. Genes involved in various metabolic processes, including low temperature signalling, are being expressed at considerable levels. At the same time, various well known cold/drought stress related genes are being induced up till high levels. Once maximal tolerance is reached, expression of most genes is lowered considerably except for the cold/drought stress related ones. This finding is seemingly in contrast with data from dormancy-level tests. It is however an indication that metabolism is very strictly pointed. At the same time our findings made clear that a different experimental approach is needed to find indicators that relate to growth potential.

6. Exploitation and dissemination of results

At present, it is not uncommon for 25% of the seedlings in new plantations to die. Poor establishment is often caused by frost damage or desiccation, during storage of insufficiently hardened plants. Therefore, better characterisation of the seedling level of cold hardiness will ensure an enhanced quality of planting stock. These genes can be used for the future development of a rapid hardiness test that will support nursery management decisions and facilitate forestation logistics.
In the development of a proof of principle assay we focused on dehydrins because precious research and early Cold Tree findings suggested that this gene family would be a good candidate for a cold tolerance assay. This development has been protected via a patent (EP 05075317.7). The chosen test format (NALFIA) is merely one of the many options. Many more formats are available and can be useful. Test format should be adapted to the needs of nurseries with respect to required information and the moment of testing. This test can be on the market in 12 months time. We are now looking for partners for validation in practice and marketing.
In addition to the dehydrins used in the test described above, many of the other selected genes can be translated into a diagnostic quality test. Future work should focus on the final selection of assay content for beech and pine, validation in practice and extension to other species (such as *Picea*). To this end several consortia of research institutes and nurseries have been formed (in Sweden and The Netherlands) or are being formed.
The combined database will aid in this future work by offering simple interfaces for queries compared to manipulating a large number of spreadsheets. In the future it will also allow other users, beyond the Cold Tree partners, to interrogate the data with their own questions in mind. Cold Tree ESTs will be entered in the Picme collection, a Plant EST Resource Centre and Repository (www.picme.at).

Cold Tree results have been widely disseminated among nursery/forestry sectors in Denmark, Sweden, UK and the Netherlands via various publications in trade journals, interviews for local broadcasting companies, posters and lectures on sector meetings and the demonstration workshops organised in the final year of the projects lifetime. Several scientific papers are in progress or submitted. Cold Tree results have been discussed repeatedly on international scientific conferences via posters and oral presentations.

7. Policy related benefits

On average two-third of the European forestland is commercially exploited and sustained-yield use of forests is standard. In 1993 at the Ministerial Conference of Forest Protection in Europe, European countries and the EU committed themselves to the principle of sustainable management. On an annual basis, millions of seedlings are necessary for reforestation and afforestation and large capitals are invested in logistics, transportation and plant material. To allow for a continued sustained-yield use of European forests, cost-effective reforestation is essential. Seedling vitality, as measured by regrowth capacity upon plantation in spring, is largely determined by the climatic, storage and transport conditions in the preceding autumn and winter. Untimely lifting and storage of insufficiently hardened plants will result in reduced vitality.
The results from the growth and field studies presented in this report improved our understanding of the response of different key species, provenances, ages, and plant types to winter climate. This will facilitate the judgement of 'well-adapted' by both suppliers and customers. The combination with transcriptional profiling led to the development of molecular tests will provide a rapid, objective, and cheap way of evaluating hardiness and dormancy. Insight in the effects of provenance, age and climatic conditions on the expression of genes related to frost hardiness will facilitate the identification of broadly applicable genes for future implementation in diagnostic tests. Such tests will aid in defining EU standards on plant condition, thereby improving forestation planning and forest tree nursery management.

Increasing internationalisation of forest tree nurseries even enhances the demand for accurate descriptors of dormancy and frost hardiness. Conditions at the nursery may differ largely from the conditions at the planting site. Also, plants from the same species but from different provenance may differ in dormancy status. To be successful in international trade nurseries need to be able to deliver seedlings that are fit to meet the conditions at the planting site. For this reason an international approach of the problem was essential.
In summary the results of this project will aid in:

- continuation of the sustained yield use of European forests by enabling cost-effective reforestation and afforestation
- maintaining and increasing the competitiveness of European tree nurseries
- promoting the use of biotechnological tools in the European forestry sector, in particular for determining plant quality

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