DEHYDRINS IN SCOTS PINE TISSUES: RESPONSES TO ANNUAL RHYTHM, LOW TEMPERATURE AND NITROGEN

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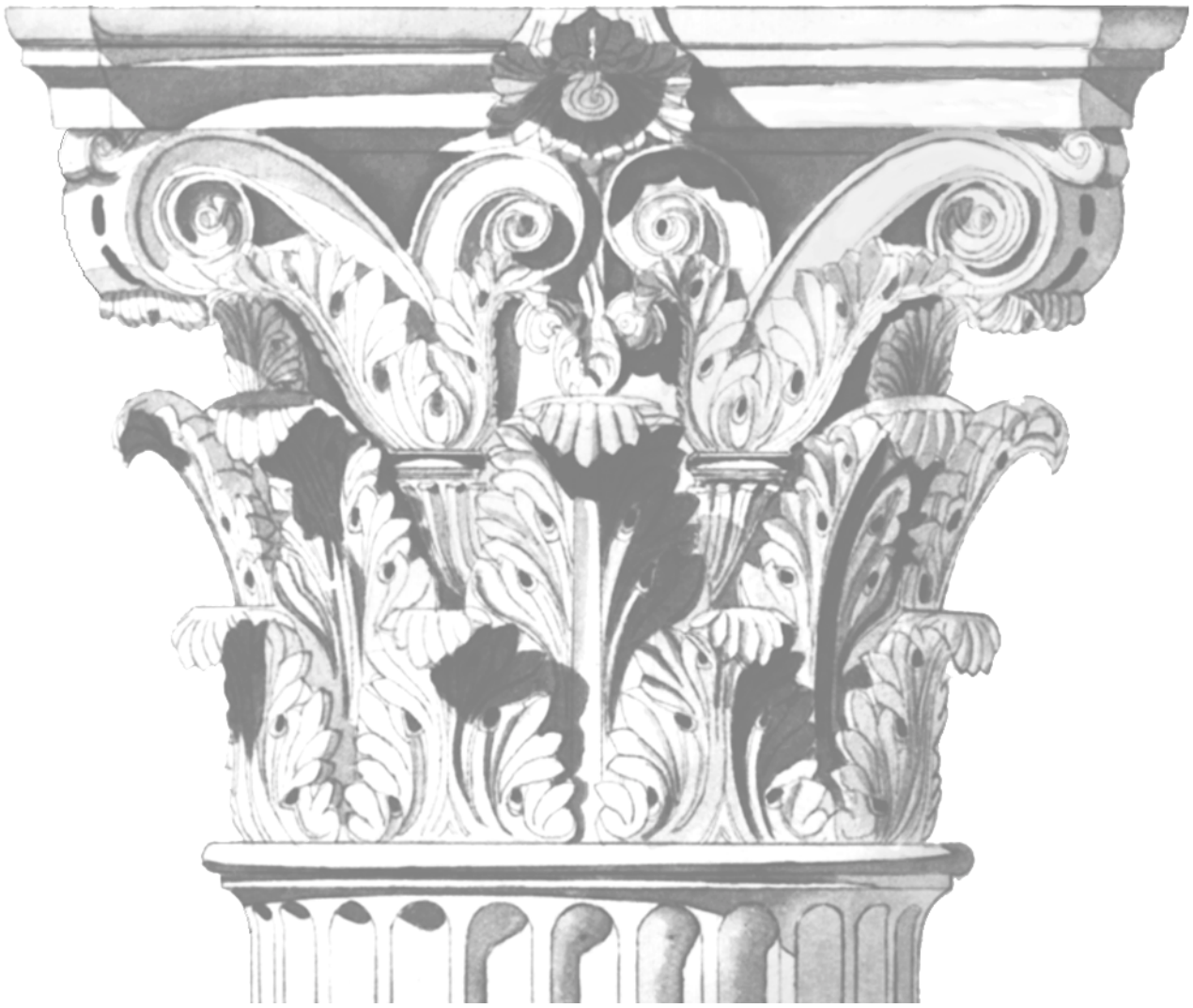

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Abstract

Natural seasonal variation and the effects of cold treatment and nitrogen fertilization on protein expression with special emphasis on dehydrin proteins, were studied using different aged Scots pine (*Pinus sylvestris* L.). Several different dehydrins were found and their expression depended on the tissue type, tree age or specific treatment. Their concentrations fluctuated seasonally and in response to nitrogen fertilization, but no effects of low temperature on the dehydrins of seedlings were observed. A 60-kDa dehydrin was associated with cold acclimation in the bud and bark tissues of mature trees and in the needles of seedlings. In the needles of mature trees, this dehydrin was associated with springtime desiccation, which was detected as a significant decrease in the osmotic potential of needles.

The quantity and quality of soluble proteins altered seasonally in Scots pine tissues, but low temperature treatment alone did not have any effect on the proteins. Soluble protein concentration increased during autumn and decreased in spring in buds and bark, but not in the needles of mature trees. In needles of seedlings, however, protein concentrations altered seasonally. Several proteins, of varying molecular weights, were more abundant in winter in all the tissues studied and some increased in concentration in the nitrogen-fertilized seedlings. The role of these proteins as a storage reserve in Scots pine is discussed.

The osmotic potential of needles showed seasonal fluctuation, being high in the summer and low during the winter. Low temperature treatment decreased the osmotic and water potential of needles and increased the concentrations of soluble sugars in seedlings. Based on carbohydrate analyses, the metabolism of seedlings acclimated to low temperature in less than ten days. Nitrogen fertilization increased the content of total nitrogen and the soluble protein concentrations in the needles of seedlings and the growth both in the mature trees and seedlings. Although the frost resistance showed no response to nitrogen-fertilization, the soluble proteins and dehydrins were affected in a manner that suggested an earlier growth resumption of spring in the fertilized trees.

Keywords: carbohydrates, cold acclimation, deacclimation, osmotic potential, *Pinus sylvestris L.*, soluble proteins, water potential.

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Punkaharju, February 2001 Sari Kontunen-Soppela

Abbreviations

ABA	abscisic acid
BSP	bark storage protein
COR	cold-regulated proteins
DTT	dithiothreitol
DW	dry weight
EDTA	ethylendiaminetetraacetic acid
FW	fresh weight
IgG	immunoglobulin G
LEA	late embryogenesis abundant
LT_{50}	the temperature causing 50% electrolyte leakage
NPK	nitrogen phosphorus potassium
PVDF	polyvinylidene fluoride
PVPP	polyvinylpolypyrrolidone
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
VSP	vegetative storage protein

List of original papers

The thesis is based on the following publications, which are referred to in the text by their Roman numerals:

I Kontunen-Soppela S & Laine K (2001) Seasonal fluctuation of dehydrins is related to osmotic status in Scots pine needles. Manuscript submitted for publication.

II Kontunen-Soppela S, Lankila J, Lähdesmäki P & Laine K (2001) Responses of protein and carbohydrate metabolism of Scots pine seedlings to low temperature. Manuscript submitted for publication.

III Kontunen-Soppela S, Hynynen J, Laukkanen H, Laine K & Lähdesmäki P (2001) Soluble protein patterns and dehydrins of Scots pine buds in long-term NPK fertilization treatment. Manuscript submitted for publication.

IV Kontunen-Soppela S, Taulavuori E, Taulavuori K, Laine K (2001) Effect of nitrogen fertilization on the protein metabolism of Scots pine seedlings during hardening. Manuscript submitted for publication.

V Kontunen-Soppela S, Taulavuori K, Taulavuori E, Lähdesmäki P & Laine K (2000) Soluble proteins and dehydrins in nitrogen-fertilized Scots pine seedlings during deacclimation and the onset of growth. Physiol Plant 109: 404-410.

Contents

1 Introduction

1.1 Cold acclimation and dormancy in woody plants

Plants that are able to overwinter in temperate and cold regions can cold acclimate; i.e. they are capable of increasing freezing resistance in a controlled manner. During the acclimation period, cold or frost hardening develops, providing the plant with a tolerance to low temperatures that would be lethal to an unhardened plant (Howarth & Ougham 1993). The degree of hardening varies in relation to the growth stage of the plant and is closely associated with the dormancy cycle (Bigras 1996). According to Doorenbos (1953), dormancy includes "all the cases when a living tissue predisposed to elongate does not do so." Frost hardening can increase independently of dormancy, but usually trees cannot attain full hardiness until growth has ceased (Weiser 1970, Bigras 1996). Because winter dormancy, or the endodormancy in the classification of Lang *et al.* (1987), and cold hardiness are overlapping events in plants, the processes involved in them are difficult to distinguish and separate.

Cold hardening in nature is suggested to be a gradual process (Weiser 1970), which in northern conifers is first induced by a shortening photoperiod (Sakai & Larcher 1987, Taulavuori *et al.* 1997). The second stage of hardening involves increasingly lower temperatures, and for some very hardy species a third stage, which requires very low temperatures, is possible (Weiser 1970). In spring, the cold hardiness of trees is progressively lost due to increasing temperatures (Dormling 1993, Bigras 1996, Taulavuori *et al.* 1996) but specific stages in dehardening have not been identified. The loss of endodormancy in spring is associated with favorable temperatures and occurs simultaneously with deacclimation, when the chilling requirement of the plant is met (Sakai & Larcher 1987, Bigras 1996). However, the metabolic activity of cells can increase before the frost hardiness of the plant is lost (Häggman *et al.* 1985, Kupila-Ahvenniemi 1985, Näsholm & Ericsson 1990).

The timing of cold hardening in plants as well as the onset of dormancy are genetically determined that are adapted to local conditions (Weiser 1970, Sakai & Weiser 1971). In the case of trees with a wide range of distribution, the timing of bud set and the development of

frost resistance can vary significantly between different provenances or ecotypes of the same species (Sakai & Weiser 1971, Sarvas 1974, Hurme *et al.* 1997, Repo *et al.* 2000).

1.2 Freezing injury and dehydration in plant cells

When exposed to low temperatures, plant cells encounter three main problems; changes in the spatial organization of biological membranes, a retardation of biochemical and chemical reactions, and alterations in the availability and status of water (Vézina *et al.* 1997). During cold acclimation plants initiate mechanisms to prevent or survive freezing injury, and an essential part of cold tolerance is the ability to recover from freeze-induced injury (Howarth & Ougham 1993). Ice formation is generally directed to the extracellular space in freezing tolerant plants, whereas in susceptible plants freezing is intracellular, causing death (Burke *et al.* 1976). During extracellular freezing, ice forms between cells, which in turn initiates the removal of free water from cells in order to come to vapor pressure equilibration. This leads to dehydrative stress in the cells (Sakai & Larcher 1987, Uemura & Steponkus 1999). Freezing injury is regarded to be a consequence of membrane lesions that are caused by the dehydration that occurs during freezing (Steponkus 1984), although other factors may also contribute to the cellular damage induced by freezing (Thomashow 1999).

Dehydration, which is caused either by freezing, drought or salt stress, also occurs during developmental events such as seed desiccation and, is thought to be analogous at the cellular level (Bray 1993). According to Levitt (1972) "…the only freezing tolerance developed by a plant is tolerance of the secondary freezing stress - the water stress induced by freezing". Crowe *et al.* (1990) regard freezing stress similar to dehydration only in that both stresses involve the removal of freezable water from cells. The two stresses differ in that the remaining bound water can only be taken away by dehydration but not lowering of the temperature. On the other hand, water stress and the phytohormone ABA are known to improve the cold tolerance of plants (Palva & Heino 1998). Evidence for the similarity of dehydration and freezing has also been raised from studies that have shown that the same proteins and genes can be induced by the different stresses that cause cellular dehydration (see references in Palva 1994, Thomashow *et al.* 1997, Thomashow 1999). However, the signaling pathways of cold and other dehydration stresses are turning out to be partly different (Shinozaki & Yamaguchi-Shinozaki 2000).

1.3 Metabolic changes related to cold acclimation and hardening

In order to tolerate the stresses that they face, plants have to adapt their metabolism. Several cellular and metabolic functions are altered by low temperatures and freezing. Because the plasma membrane is thought to be the primary site of injury during freezing (Levitt 1972), most of the alterations are aimed at preserving the integrity of membranes (Uemura & Steponkus 1999).

Plant membranes undergo both qualitative and quantitative modifications during periods of cold acclimation and deacclimation. The lipid composition of the plasma membrane and chloroplast envelopes change during cold acclimation in a way that the threshold temperature of membrane damage is lowered compared to non-acclimated plants (Uemura & Steponkus 1999). This is due to the increasing fluidity of the cold acclimated membranes, which results from a change in lipid composition towards an increase in desaturated fatty acids (Sutinen 1992, Vogg *et al.* 1998). Because of the alterations in lipid components of membranes, the protein fraction in them also changes. The lipid-protein ratio of thylakoid membranes increases during cold hardening (Vogg *et al.* 1998) as well as the activity of plasma membrane H+-ATPase (Hellergren *et al.* 1983, Sutinen 1992). Yoshida *et al.* (1999) reported changes in tonoplast enzymes during a low temperature treatment. The total lipid concentration also increases in conifer needles during winter (Fischer & Höll 1991).

Carbohydrate content is known to vary according to the hardening status of a tissue. Starch concentrations decrease and the concentrations of soluble sugars increase in cold acclimating tissues of woody plants (Aronsson *et al.* 1976, Fischer & Höll 1991, Ögren *et al.* 1997, Greer *et al.* 2000). The oligosaccharides raffinose and stachyose are especially associated with cold hardiness, low temperature and dormancy, but sucrose also enhances cold hardiness and desiccation tolerance of buds in woody plants (Stushnoff *et al.* 1997). In addition to carbohydrates, woody plants accumulate other solutes, such as proline (Jouve *et al.* 2000) or glutamic acid (Pietilä *et al.* 1991), when exposed to low temperatures or during natural hardening. In general, the function of the solutes is to maintain turgor in dehydrating cells, but they may also have protective effects on macromolecules (Smirnoff 1998). Cold temperatures also lead to oxidative stress in plants by inducing reactive oxygen species. Therefore, the activities of antioxidant enzymes (ascorbate peroxidases, glutathione reductase, superoxide dismutase, etc.) taking part in the scavenging of free radicals, as well as the levels of antioxidant compounds, are induced during the cold acclimation (Tao *et al.* 1998, Taulavuori *et al.* 1999a) and play an important role in recovery from freeze-induced damage.

The process of adaptation to low temperatures may cause changes in the function of genes and proteins. The adaptation can involve the modification of pre-existing proteins and the up- and down-regulation of gene expression or protein synthesis. New gene expression and protein synthesis has also been observed during the adaptation. It is suggested that cold induced gene activity may aid in the metabolic adjustment to low temperatures or confer freezing tolerance to tissues (Guy 1990). Stress-induced genes may also be involved in the signal transduction of the stress-response (Ingram & Bartels 1996, Thomashow 1999). Many cold-induced proteins and genes have been studied in several plant species (see references in Guy 1990, Howarth & Ougham 1993, Hughes & Dunn 1996, Thomashow 1999). These proteins include apoplastic proteins having antifreeze activity (Griffith *et al.* 1992), cryoprotective proteins (Hincha *et al.* 1990) and molecular chaperones (Guy *et al.* 1998). However, the function of many cold-induced proteins is not yet known.

In woody plants, many of the genes and proteins related to cold acclimation may also be connected to the dormancy status of the plants (Wisniewski *et al.* 1996, Rowland & Arora 1997). In general, cold induced proteins in woody perennials can be divided into two

groups, proteins related to desiccation tolerance and storage proteins which serve as an overwintering form of nitrogen reserves.

1.3.1 Dehydrins

A group of dehydration-related proteins and their genes were first detected from developing seeds and categorized as Late Embryogenesis Abundant (LEA) proteins (Delseny 1994). LEA proteins are divided into groups based on specific amino-acid sequences (Bray 1993 and 1994, Dure 1993). The D-11 family (Dure 1993) or group 2 (Bray 1993) LEA proteins are called dehydrins. Dehydrin proteins are induced in plants by dehydration-related environmental stresses such as low temperature, drought or high salinity (Close 1996) and some studies also report a response to wounding (Rouse *et al.* 1996, Richard *et al.* 2000). These proteins, of variable molecular masses, have been found in many plant species. Indicative of dehydrins is the presence of one or several lysine-rich units called the K-segments conserved near the carboxy terminus of the protein and repeated several times throughout the sequence (Close 1996). Some dehydrins also possess a string of serine residues (S-segment). Another consensus sequence (DEYGNP), the Y-segment, can be found near the amino terminus of most of the dehydrins (Close 1996). The 15-amino-acid consensus sequence of the Lys-rich motif EKKGIMDKIKEKLPG has been used for antibody production (Close *et al.* 1993). Several proteins from different species, including woody perennials, have been detected with this anti-dehydrin antibody (Close *et al.* 1993, Wisniewski *et al.* 1996, Sauter *et al.* 1999). In conifers, dehydrin proteins and genes have been identified from the seeds of *Pinus* (Close *et al.* 1993) and *Pseudotsuga* (Jarvis *et al.* 1996), but studies on gene expression are restricted to needles of *Picea glauca* seedlings (Richard *et al.* 2000).

Dehydrins are localized to the cytoplasm or nucleus, depending on the cell type (Close 1996). Recently dehydrin-like proteins have been found in storage protein bodies and amyloplasts of cold acclimating *Betula pubescens* (Rinne *et al.* 1999) and plastids of *Prunus persica* bark (Wisniewski *et al.* 1999). Cold acclimation also induces their accumulation in the mitochondria of several plant species (Borovskii *et al.* 2000). Some of the dehydrins are post-translationally regulated by glycosylations (Golan-Goldhirsh *et al.* 1998, Levi *et al.* 1999) and phosphorylations (Vilardell *et al.* 1990), the latter of which is related to the targeting of these proteins to nucleus (Jensen *et al.* 1998).

The function of dehydrins is suggested to be the preservation of the structural integrity of cells by inhibiting the coagulation of macromolecules (Close 1997) but direct evidence for the role of dehydrins in the cold tolerance of plants also exists. By overexpressing constitutively a gene that regulates COR (cold-regulated)-proteins, some of which are dehydrins, Jaglo-Ottosen *et al.* (1998) showed that COR-genes promote the freezing tolerance in *Arabidopsis*. The peach (*Prunus persica*) PCA60 dehydrin possesses antifreeze activity thereby altering the shape of the ice crystals, which could aid in reducing the freezing damage to the cells (Wisniewski *et al.* 1999). Cryoprotective activity of dehydrins on enzyme function has been demonstrated with the *Prunus persica* dehydrin (Wisniewski *et al.* 1999) and the birch (*Betula pubescens*) RAB-16-like dehydrins (Rinne *et al.* 1999).

Other possible functions of dehydrins include molecular chaperone-like properties (Close 1996) and the preservation of membrane integrity during dehydration (Close 1997).

1.3.2 Storage proteins

Storage proteins that are present in the nonseed tissues of plants are called vegetative storage proteins (VSP) or bark storage proteins (BSP) and they are important in the cycling of nitrogen in plants (Staswick 1990). The amount of VSP increases in the bark and wood of dormant trees during autumn, especially in deciduous trees (Wetzel *et al.* 1989, Sauter & van Cleve 1990, Langheinrich & Tischener 1991, Arora *et al.* 1996) but also in conifers (Wetzel & Greenwood 1989). VSPs are used for new growth in spring. The leaves of poplar (Lawrence *et al.* 1997, Beardmore *et al.* 2000) and the buds of interior spruce (Binnie *et al.* 1994) are also reported to contain VSPs. VSPs have been studied intensively on *Populus*, where they are reported to be glycosylated proteins (Langheinrich & Tischener 1991) encoded by a multigene family (Coleman *et al.* 1992). They are localized in protein storage vacuoles (protein bodies) in stem tissues (Wetzel *et al.* 1989). Short photoperiods (Coleman *et al.* 1992) and low temperature (van Cleve & Apel 1993) induce VSPs in poplar and their synthesis is controlled by the availability of nitrogen (Coleman *et al.* 1994) and the hormone methyl jasmonate (Beardmore *et al.* 2000). The poplar VSPs exhibit homology to poplar seed storage proteins (Beardmore *et al.* 1996) and wound-induced proteins (Davis *et al.* 1993) but not to other seed storage proteins.

1.4 Effects of excess nitrogen on plants

Plant growth in boreal forest ecosystems is generally limited by the availability of nitrogen. The forms of nitrogen in the soil available to plants are usually regarded to be nitrate (NO_3^-) and ammonium (NH_4^+), the latter of which is the preferred form of nitrogen absorbed by Scots pine (Flaig & Mohr 1992, Gezelius & Näsholm 1993). Forest plants are also capable of obtaining nitrogen as organic substances (Näsholm *et al.* 1998), with the help of mycorrhiza (Abunizadah & Read 1986 a and b, Abunizadah *et al.* 1986, Finlay *et al.* 1992). Plants can also sequester nitrogen from wet and dry atmospheric deposition via leaves as in oxidized (NO_x) or reduced (NH_3 or NH_4^+) form (Rennenberg & Gessler 1999).

Nitrogen promotes growth and increases biomass production of trees and nitrogen fertilization has been used to increase wood production and forest growth. In excess, nitrogen may have adverse effects on the vitality of plants. Nitrogenous air pollutants and fertilizers cause changes in the shoot/root ratio (Van der Eerden & Perez-Soba 1992, Entry *et al.* 1998) and reduce mycorrhizal induction in Scots pine (Väre 1989, Perez-Soba *et al.* 1995, Wöllecke *et al.* 1999). The reduced activity of roots can create a nutrient imbalance (van Dijk & Roelofs 1988, Ferm *et al.* 1990, Jokela 1998). These factors may consequently increase the susceptibility of trees to stresses caused e.g. by frost, drought and pathogens (Bobbink *et al.* 1992, Fangmeier *et al.* 1994, Wöllecke *et al.* 1999).

Growth disorders and mortality of apical buds in Scots pine are observed under high ammonium supply and these have been attributed to decreased frost tolerance of the trees (Ferm *et al.* 1990, Pietilä *et al.* 1991). Reports concerning the effect of nitrogen on frost resistance vary depending on the species and the form of nitrogen (NOx, NH_3). Nitrogen has been reported to both impair (Hellergren 1981, Dueck *et al.* 1990, Cape *et al.* 1991) and increase the freezing resistance of conifer needles during cold hardening (DeHayes *et al.* 1989, L'Hirondelle *et al.* 1992). There are also studies, where frost resistance of trees is not affected either way by nitrogen (Wiemken *et al.* 1996, Taulavuori 1998). Nitrogen-rich fertilization is known to prolong the growing period of plants and thus increase the susceptibility of trees to late and/or early frosts (Cannell & Sheppard 1982, Margolis & Waring 1986, Bobbink *et al.* 1992). According to Jokela (1998) nitrogen fertilization increases the mesomorphism of Scots pine needles and may thus lower natural protection to environmental stresses. Excess nitrogen, either as fertilization or ammonium deposition, has also caused frost-drought-type injuries in plants (Jalkanen & Aalto 1993, Gordon *et al.* 1999).

Extra nitrogen supply alters the metabolism of nitrogenous compounds in plants and an increased concentration of amino acids (Pietiläinen & Lähdesmäki 1986, Zedler *et al.* 1986, Ferm *et al.* 1990, Näsholm & Ericsson 1990, Näsholm *et al.* 1994), soluble proteins (Zedler *et al.* 1986, Ferm *et al.* 1990), and polyamines (Taulavuori *et al.* 1999b) have been reported. The activities of enzymes involved in N uptake are also induced in nitrogen-supplied Scots pine (Perez-Soba *et al.* 1994). Excess nitrogen also changes protein patterns in Scots pine needles during acclimation and deacclimation (Pietilä *et al.* 1991). Because proteins play an important role in the development of freezing tolerance, it has been suggested that the observed changes may affect the frost hardiness of nitrogen-supplied trees.

2 Aim of the research

The main objectives of this study were to gain an understanding of the effects of low temperature, nitrogen, and seasonal changes on the metabolism of Scots pine. Research was focused on a group of proteins that are induced by cellular dehydration, the dehydrins. The dehydrins were studied to characterize the seasonal variation, their presence in different tissues, their induction by low temperature or nitrogen and their relation to the water status of needles. Other proteins that could be related to the cold hardiness of Scots pine were also studied. The response of these other seasonally fluctuating proteins to nitrogen fertilization was assessed.

Different experiments were conducted in order to

- describe the seasonal variation in water relations, soluble proteins and dehydrins in different tissues and in trees of different ages (I, III-V)
- determine the effect of a low temperature treatment on water relations and carbohydrate and protein metabolism in seedlings (II)
- investigate the effect of timing and dose of nitrogen fertilization on growth, patterns of proteins and dehydrins (III-V), osmotic potential (V) and cold resistance (IV, V) of mature trees (III) and seedlings (IV, V)

3 Materials and methods

3.1 Plant material and growing conditions

Branches of mature trees (I, III) or whole seedlings (II, IV, V) of Scots pine (*Pinus sylvestris* L.) were collected and stored on ice. The needles, buds or living bark were dissected in the laboratory, frozen immediately in liquid nitrogen and stored at −70 °C until analyzed. The material used and the experiments conducted are characterized in detail in Table 1.

Table 1. Plant material and experiments used in the studies.

Plant	Origin	Tissue used in the sampling	Experiment	Study
Mature trees 40-year-old	Natural stand local seed origin, Oulu (65° 03'N; 25° 27'E)	needles buds bark	Seasonal changes	I
Mature trees 40-year-old	Seeded stand local seed origin, Muhos (64º 51'N; 26º 17'E)	buds	NPK-fertilization 150 kg N ha^{-1}	III
2-year-old seedlings	Nursery seedling seed origin Utajärvi (65º 45'N; 26º 23'E)	needles	Low temperature treatment	II
2-year-old seedlings	Nursery seedling, seed origin Pyhäntä (64º 05'N; 25º 20'E)	needles	N-fertilization 442 or 884 kg N ha^{-1} in June or July	IV
2-year-old seedlings	Nursery seedling seed origin Utajärvi (65º 45'N; 26º 23'E)	needles	N-fertilization 442 or 884 kg N ha^{-1} in July	V

To assess seasonal changes in Scots pine metabolism, mature trees growing in a natural stand in Oulu, were used for sampling (I). Samples were collected for analyses monthly between 12 October 1998 and 13 September 1999. The annual growth of the previous year was harvested from October 1998 to June 1999 and the new growth between July-September, 1999.

The effect of low temperature was studied on two-year-old Scots pine (II). The seedlings were raised in paper-pots and overwintered out-of-doors. The seedlings were potted into sand and kept outside under field conditions at the beginning of the growth period. After an adaptation period of two days at 18 ºC in 70% relative humidity in growth chambers, half of the seedlings were exposed to cold treatment (4 ºC) for 14 days in the chambers in a 16-h photoperiod. Control plants were held at 18 ºC at the same photoperiod.

The long-term effects of nitrogen fertilization on seasonal changes in protein metabolism were studied on 40-year-old mature Scots pine (III). Fertilization with 150 kg N ha^{-1} had been repeated every five years using nitrogen-rich NPK fertilizer 'Super Y' (ratio of nutrients 20-4-8). Control samples were taken from an unfertilized plot.

Nitrogen fertilization effects were also studied on two-year-old Scots pine seedlings. The pot seedlings were planted to pots in May 1996 (IV) and June 1997 (V) in a mixture of sand, peat and raw forest humus (1:1:1) and inserted into a sand bed in the experimental field (Taulavuori *et al.* 1999b). Ammonium nitrate ($NH_4NO_3xCa(OH)_2$)-fertilizer was given at either 442 or 884 kg N ha^{-1}. In experiment IV, the fertilization was done either on June 6 or July 6, 1996, and in experiment V, on July 7, 1997. Unfertilized seedlings were used as controls in both treatments. Seedlings were collected for samples during the autumn 1996 (IV) and in the spring of 1998 (V).

3.2 Protein analyses

Needles of current (I, IV) or previous year (I, II, V), buds (I, III) and living bark (I) were used for protein extractions.

For the analyses of bud soluble proteins in experiment III, frozen buds were ground in liquid nitrogen and homogenized in an extraction buffer containing 0.1 M Tris-HCl (pH 9.0), 1.5% (w/v) PVPP, 5 mM DTT and 2 mM EDTA. Proteins were precipitated with 70% ammonium sulphate and the precipitates dissolved in distilled water. The samples were desalted with Econo-Pac 10DG columns (Bio-Rad), concentrated by lyophilization and redissolved in distilled water. Protein concentrations were measured with the bichinconinic acid method (Smith *et al.* 1985). Proteins were diluted in SDS-PAGE sample buffer (Laemmli 1970).

For the bud, needle and bark protein analyses in experiments I, II, IV and V, frozen, powdered tissue (0.5-1 g) was homogenized in extraction buffer (50 mM Na-borate, 50 mM ascorbic acid, 100 µM DTT, pH 9.0) and 50% (w/w) PVPP. Protein concentrations of supernatants were measured according to Bradford (1976) and equal amounts of protein were precipitated with 0.015% (v/v) Na-deoxycholate and 7.2% (v/v) TCA. The precipitate was washed with acetone, centrifuged, and the resulting pellets were dissolved in SDS-PAGE sample buffer (Laemmli 1970).

Proteins obtained with both extraction methods were separated by SDS-PAGE (Laemmli 1970). The gels were stained with colloidal Coomassie Blue G-250 according to Neuhoff *et al.* (1988). The immunoblot analysis was conducted according to Harlow & Lane (1988). Proteins were transferred to Immun-blot-PVDF (Bio-Rad) or supported nitrocellulose membrane. A polyclonal antibody against the conserved region of the dehydrin-family (Close *et al.* 1993) was used at a 1:750 or a 1:1000 dilution. A negative control was made by blocking the antibody with the same peptide that was used for producing it. A 1:5000 dilution of second antibody (goat-antirabbit IgG alkaline phosphate conjugate, Sigma) was applied and the membrane was developed with an alkaline phosphatase conjugate substrate kit (Bio-Rad).

Protein contents of replicate gels and blots were analyzed and their protein contents were quantified with Fluor-S™ MultiImager System (Bio-Rad, Hercules, CA, USA). In order to compare the amount of the 60-kDa dehydrin between different months (I), the band volumes in the immunoblots were scanned.

3.3 Osmotic potential, water potentials, and dry weight

The osmotic and the water potentials of the needles were measured with a dew point meter (HR-33T, Wescor Inc., Logan, UT, USA) (I-II, V). For measurement of the osmotic potential, detached needles were frozen in liquid nitrogen and stored at −70 ºC. The osmotic potential of fluid pressed from the needles was measured the next day according to the manufacturer's instructions. The water potential of 5-mm needle pieces was measured immediately after the needle dissection.

The dry weight of needle samples was measured after drying 24 h in 80ºC (V). The relative dry weight was calculated as DW/FW x 100%.

3.4 Frost resistance

Frost resistance of needles was measured according to Taulavuori *et al.* (1996, 1997) (V). The viability of needles was determined by the relative electrolyte leakage method. Frost resistance was expressed as the temperature causing 50% electrolyte leakage (LT_{50}), which was calculated using a non-linear logistic function.

3.5 Carbohydrate analyses

Soluble sugars and starch contents of dried (80 ºC, 24 h) needles were quantitatively determined using the anthrone test (Hansen & Møller 1975, Antikainen & Pihakaski 1994) (II).

3.6 Measurements of growth and nitrogen content

The length of apical buds of the main shoot, and later on the length of the growing new shoot, was measured every second day during the height growth period, then less frequently later in the growth period (V). The mean diameter and height growth of the mature trees as well as some other stand and tree characteristics were measured at 5-year intervals (III).

Total nitrogen and carbon contents of the dried needle samples were analyzed using a CHNS analyzer (Automatic Elemental Analyzer EA1110, Fison Instruments S.p.A., Milan, Italy) (V).

3.7 Statistical analyses

The statistical analyses for testing the effects of the different treatments were performed using a one-way ANOVA (I, II), two-way-ANOVA followed by Sheffé's paired comparison (III, V) and an analysis of covariance (ANCOVA) (IV). The correlation in osmotic potentials and relative dehydrin levels was calculated using the non-parametric Kendall's correlation test (I).

4 Results

4.1 Soluble protein concentrations

The concentration of soluble proteins in the needles of Scots pine seedlings increased during the autumn period of cold acclimation (IV), but no significant differences were found in the needles of mature trees during the autumn (I). In spring, the protein concentration of needles both in mature trees and seedlings were stable (I, V), except for seedlings that were fertilized with a nitrogen dose of 884 kg N ha^{-1} (V). A transient decrease in the needle protein concentrations was observed in the 884 kg N ha^{-1} fertilized seedlings in late April-May (V). The low temperature treatment did not have an effect on the concentration of soluble proteins in the needles (II). However, the concentration of soluble proteins in the needles of the cold-treated seedlings increased 7 and 10 days after the beginning of the low temperature treatment (4 ºC).

The soluble protein concentration of buds in mature trees decreased during the spring (I, III), even though the concentration increased temporarily prior to the bud break (I). The bud protein concentrations were lowest during the summer and increased in autumn towards the winter (I, III). Bark soluble protein concentration exhibited an increasing trend from July to March (I). The bark protein concentration decreased in June prior to budbreak, and was at its lowest during the summer (June-August) (I).

Summer fertilization significantly affected soluble protein concentration in needles collected in the following spring (V), but an increase was found in the autumn following the summer fertilizations in seedlings receiving the higher dose of fertilization (884 kg N ha^{-1}) (IV). There was no effect of the NPK-fertilization on the concentrations of bud soluble proteins (III).

4.2 Protein patterning in SDS-PAGE

Patterns of soluble proteins varied seasonally (I, III-V) and in response to nitrogen fertilization (III-V). The seasonal variation of proteins was different in the different tissue

types (needles, buds and bark) (I). SDS-PAGE profiles of needle proteins showed no changes during the cold treatment (II).

The concentration of several polypeptides was higher during winter-spring than in the growth period (I, III-V) and the increase in the relative amount of these protein components was especially marked in bark and bud tissues (I). The molecular masses of the proteins that change in response to season in different tissues are presented in Table 2.

Table 2. Soluble proteins in different tissues of Scots pine that accumulate during cold acclimation in autumn (I, III, IV) or decrease during deacclimation in spring (I, III, V). Proteins that increase in concentration due to N fertilization are marked with asterisks. The numbers indicate the molecular masses of proteins in kDa.

Tissue	Needles			Buds		Bark
Experiment	I	IV	V	I	III	I
	147			147	147	147
				44		
					40	
	37					
		35*				
	32		32*	32		32
					30	
				29		29
	27			27		
	25	25*	25*		25	
	23					
						22
				21		21
		20*	20*		20	
	19			19		19
					18	
			17*			

An effect of nitrogen fertilization was observed in needles by the increased concentrations of specific protein components (Table 2) (IV-V). In buds, no significant alterations in the amount of proteins in the gels were observed due to NPK fertilization (III). Some proteins (18, 20, 25, 30, 40 and 147 kDa) that fluctuated in amount in the buds seasonally, disappeared from the trees in the NPK-fertilized area in late spring, but were still seen in the buds of unfertilized trees (III).

4.3 Dehydrins

Several dehydrin proteins of different molecular weights were detected in Scots pine tissues (Table 3). Their presence and abundance were dependent on the age of the material (seedling vs. mature tree), season, and the tissue studied.

Table 3. Dehydrin profiles in different Scots pine tissues. The numbers indicate the molecular masses of proteins in kDa.

Tissue	Needles	Buds	Bark
Experiment	I, II, IV, V	I, III	I
		73	
	70	70	
	60	60	60
	56	56	56
	50	50	50
		48	
	47		
	42		
		40	
		37	

In bud and bark of mature trees, and in the needles of seedlings, a 60-kDa dehydrin was most abundant during the winter (I, IV-V) and its content decreased in the spring. The amount of this dehydrin did not markedly increase in autumn in the needles of mature Scots pine, but an increase was observed during the springtime in March-May (I). The needle dehydrins of seedlings did not show any response to low temperature treatment (II), and both in the needles of seedlings and mature trees, no increase in dehydrin content was observed during the hardening period (I, IV).

The number of proteins detected by the dehydrin antibody increased during deacclimation in the spring in all tissues (I, III, V), but the band intensities were low when compared to the intensity of the 60-kDa dehydrin. The 56 and 50-kDa dehydrins were present only during the growth period. A 70-kDa dehydrin appeared in buds before bud break in the experiment I, whereas a dehydrin of the same size was detected during winter in the bud samples of NPK-fertilized pines (III).

Nitrogen fertilization reduced the amount of dehydrins in the needles of seedlings during the acclimation (IV), as well as during the deacclimation period (V). There were more dehydrins of different molecular weights in the needles of fertilized seedlings in autumn than in unfertilized seedlings (IV). The dehydrin content of buds decreased earlier in the fertilized trees in late spring than in the unfertilized trees but, in autumn, the amount of the 50, 48 and 40 kDa dehydrins was lower in the buds of the unfertilized trees (III).

4.4 Water relations

The osmotic potential of needles varied seasonally both in the seedlings and mature trees (I, V). In mature trees, osmotic potentials were lowest during the spring, while the osmotic potential increased during deacclimation in the seedlings (V). Cold treatment decreased the osmotic and water potentials of the seedling needles significantly on the 4th day following the low temperature treatment, and thereafter (II). Nitrogen fertilization did not have any effect on the osmotic potentials of needles in spring (V). The relative dry weight of seedling needles was constant during the dehardening period until 19 May, after which it started to increase (V).

4.5 Frost resistance

The frost resistance of pine needles increased during hardening in autumn (Taulavuori 1998, IV) and decreased during the deacclimation period (V). Frost resistance in this study was not affected by the nitrogen fertilization treatments (Taulavuori 1998, IV, V).

4.6 Carbohydrates

Cold treatment increased the concentration of soluble sugars in the needles. The increase was evident the first day after the beginning of the cold treatment (II). Although the sugar concentration started to decrease in the middle of the cold treatment, it stayed higher in the seedlings at 4 ºC than in the control seedlings. The concentration of soluble sugars of needles remained stable in the control seedlings.

The concentration of starch decreased significantly in the cold-treated needles until the day 7, after which it began to increase (II). The concentration of starch in the control seedlings at 18 ºC was stable at first, but after day 4 the concentration decreased to a level where it remained for the rest of the experiment. On day 10 there were no differences between the treatments, but on day 14 the concentration of starch was significantly higher in the seedlings at 4 ºC than in the control seedlings.

4.7 Growth and CN-analyses

The height growth of mature trees was not affected by NPK-fertilization but the diameter growth of the fertilized trees was significantly greater (III). Fertilization almost doubled the height growth of the apical bud in the nitrogen-fertilized seedlings, irrespective of the amount nitrogen given, and the growth of terminal buds started earlier in the fertilized seedlings than in the unfertilized control seedlings (V).

Fertilization increased the total nitrogen content in the needles. Nitrogen contents ranged from 0.8-1.2% N g^{-1} DW in the unfertilized plants, and from 1.7-2.3% to 2.2-2.8% in the seedlings fertilized with 442 and 884 kg N ha^{-1}, respectively (V). The carbon content of needles was 49-50% of DW in all the treatments (V). In experiment IV, the nitrogen content of needles increased due to the dose and delayed timing of the fertilization (Taulavuori 1998).

5 Discussion

5.1 Responses to season and cold treatment

5.1.1 Soluble protein concentration and patterns

Seasonal variation in protein quantity and quality has been studied in several woody plant species. Soluble protein concentrations of pine needles increase during cold acclimation (Pomeroy & Siminovitch 1970, Näsholm & Ericsson 1990, Nozzolillo *et al.* 1990, Sutinen 1992), and decrease during the growth period (Pomeroy & Siminovitch 1970, Wetzel & Greenwood 1989). The results obtained in the present study partially differ from this previous research. The soluble protein concentration of needles did not vary between different months in the mature Scots pines (I) or in the seedlings (V) during deacclimation although an increase in the needles of the seedlings was found in late autumn (IV). Because the previous year's needles are used as storage sites for mineral nutrients, amino acids and proteins in pines (Fife & Nambiar 1984, Gezelius 1986, Wetzel & Greenwood 1989), seasonal changes in protein concentration are thought to result from the retranslocation of nitrogen within the tree (Pomeroy & Siminovitch 1970, Wetzel & Greenwood 1989). The stem of Scots pine can also act as a sink for the storage of nitrogen (Gezelius 1986). This is supported by the observation that the soluble protein concentration of bark started to decrease at the onset of growth in June and increased again during July-September (I).

The concentration of soluble proteins in buds increased simultaneously with hardening in the autumn (I, III) and decreased during deacclimation (III) in accordance with Pietiläinen & Lähdesmäki (1986). The decrease in protein concentration was observed from March (I) or April (III) onwards, when the metabolic activity of buds has been reported to increase (Hohtola *et al.* 1984, Häggman *et al.* 1985, Kupila-Ahvenniemi 1985). The resumption of shoot growth in April-June may explain the decrease in protein concentration in the buds at that time period (I, III).

In evergreen species, seasonal differences in protein quality have been shown in the bark, buds and needles (Wetzel & Greenwood 1989, Pietilä *et al.* 1991, Roberts *et al.* 1991,

Binnie *et al.* 1994, Ekramoddoullah & Taylor 1996). Changes in protein composition have been also reported in the bark, wood, and bud proteins of deciduous trees (Wetzel *et al.* 1989, Arora *et al.* 1992, Arora *et al.* 1996, Wisniewski *et al.* 1996, Rinne *et al.* 1998, Sauter *et al.* 1999). Some of these seasonally changing proteins in woody plants have been characterized as storage proteins (Wetzel & Greenwood 1989, Wetzel *et al.* 1989, Arora *et al.* 1992), photosynthesis-related proteins (Ekramoddoullah & Taylor 1996), dehydrins (Wisniewski *et al.* 1996, Rinne *et al.* 1998, Sauter *et al.* 1999), and heat-shock proteins (Wisniewski *et al.* 1996). A distinctive seasonal pattern of proteins was seen in the needles of seedlings (IV, V) and in the buds, bark and needles of mature Scots pines (I, III). The proteins that altered in amount in the course of the year appeared to be similar in their molecular weights in different tissues. The most characteristic changes were seen in the bark and bud proteins of mature trees at the onset of growth (I). Several protein components on the SDS-PAGE gels decreased in amount compared to the winter. One function for the proteins that fluctuate in amount in response to the annual rhythm in trees is suggested to be the storage of nitrogen (Coleman & Chen 1996), that is used for the new growth in spring. Bark is the main nitrogen storage organ during dormancy in deciduous trees, but conifers are also known to accumulate VSPs in bark tissues (Wetzel & Greenwood 1989). Proteins that showed seasonal fluctuation in bark (I) were of similar molecular mass as the needle proteins of seedlings (IV, V) that increased in concentration due to nitrogen-fertilization. Thus it may be that these proteins serve as a source for nitrogen reserves in both needle and bark tissues of Scots pine.

The difference between the patterns of needle proteins of seedlings (IV, V) and mature trees (I) was greater than the difference between the needle, bud and bark tissues in mature trees (I). Maturation is known to alter the metabolism of woody plants, so that differences between the adult and juvenile plant have been found e.g. in the metabolism of carbohydrates, phenolic compounds, polyamines and hormones (see references in Haffner *et al.* 1991). The needle characteristics in conifers, such as needle form, pigment content, surface waxes and CO_2 fixation (Hutchinson & Greenwood 1991), and the proteins of deciduous trees (Snowball *et al.* 1991, Hand *et al.* 1996) have also been reported to change in association with maturation.

5.1.2 Dehydrins

The accumulation of dehydrin proteins and their transcripts are induced in plant tissues by low temperature stress (Lång *et al.* 1989, Guy *et al.* 1992, Welin *et al.* 1994). In woody plants, dehydrins are also induced during the natural cold hardening period in autumn and their amounts decrease in spring when dehardening of tissues takes place (Wisniewski *et al.* 1996, Golan-Goldhirsh *et al.* 1998, Rinne *et al.* 1998, Sauter *et al.* 1999). However, in the present study, no significant increase in dehydrin content was observed in the needles of Scots pine during the cold hardening period (I, IV), nor did the cold-treated seedlings induce dehydrin synthesis (II). Some dehydrin proteins are also constitutively present in wood tissues of trees (Wisniewski *et al.* 1996). Dehydrin proteins of different sizes

appeared in both cold acclimated and deacclimated Scots pine tissues (I, III-V), but some dehydrins were found in all samples irrespective of the season or treatment (I-V).

A 60-kDa dehydrin exhibited seasonal changes and was also associated with times when the cold or dehydration stress was severe. This dehydrin was detected in high amounts in both buds and bark of mature trees during the winter (I), as well as in the needles of deacclimating seedlings (V), where its content decreased during the spring. The 60-kDa dehydrin was seen in the needles of fertilized seedlings only in August (IV). The content of this dehydrin did not markedly increase in the needles of mature Scots pine in autumn, but an increase was observed during the springtime in March-May (I). The dehydrins and other low temperature induced proteins are reported to be regulated by osmotic stress (Guy *et al.* 1992, Wang & Cutler 1995) and the increase in the amount of the 60-kDa dehydrin was associated with the lower osmotic potential in the needles (I). Changes in temperature have also induced transient changes in dehydrin protein patterns in *Populus* (Sauter *et al.* 1999). This could explain the observed decrease in the amount of the 60-kDa dehydrin in March, which was seen both in the bud and bark tissues of mature trees (I).

In addition to the 60-kDa dehydrin, several other dehydrins were detected in bud, bark and needle tissues. Most of these were seen during the spring (I, V) or autumn (I, IV) and their appearance was related to the decrease of the 60-kDa dehydrin in spring. This suggests that the observed dehydrins may be degradation products of the 60-kDa dehydrin. The other dehydrins were also seen in the cold-treated seedlings as well as in the deacclimated, growing seedlings in the control treatment (II).

The dehydrin content in the buds of mature Scots pine decreased in spring (I) until April-May when an increase was observed (I, III). The increase may have been a result of budbreak and the subsequent lack of a protective sheath around the emerging needles, as was suggested by Richard *et al.* (2000). Young, developing needles may suffer from dehydrative stress since the wax layers of young needles are not fully developed. In experiment III, the amount of different dehydrins in the buds also increased in spring. The difference in the molecular weights of the bud proteins in experiments I and III is most likely due to the different extraction methods used.

Dehydrins in needles of Scots pine seedlings were not induced by low temperature treatment (II), which is in contrast with many reports obtained from herbaceous plants (Lång *et al.* 1989, Guy *et al.* 1992, Welin *et al.* 1994, Bravo *et al.* 1999). Dehydrin genes or proteins in the seedlings of other *Pinus*-species have not shown any response to drought stress either (Chang *et al.* 1996, Costa *et al.* 1998). On the other hand, the dehydrin genes of *Picea glauca* needles can be activated with cold treatment in 8 hours and a significant accumulation of the transcript was observed in 48 hours, but the level of dehydrin proteins was not measured (Richard *et al.* 2000). The lack of response may be due to the age of the plants, since in *Rhododendron*, juvenile plants accumulated a lower level of dehydrins and also had a lower freezing tolerance than mature plants (Lim *et al.* 1999). A reduced response of a dehydrin gene expression has also been observed in water-stressed sorghum seedlings when compared to the mature plants (Wood & Goldsbrough 1997).

Although Scots pine shoots can achieve significant freezing resistance in response to low temperature alone (Christersson 1978, Smit-Spinks *et al.* 1985), shortening photoperiods also trigger cold acclimation in Scots pine (Taulavuori *et al.* 1997). Because some dehydrins are connected to seasonal changes, variation in daylength may also have an

effect on the regulation of dehydrins. Photoperiod can regulate dehydrins in *Betula pubescens* (Welling *et al.* 1997, Rinne *et al.* 1999), and some dehydrin transcripts in *Helianthus annuus* have been reported to exhibit diurnal fluctuation (Cellier *et al.* 2000).

5.1.3 Water relations and carbohydrates

Extracellular freezing causes withdrawal of water from cells (Sakai & Larcher 1987, Uemura & Steponkus 1999). The accumulation of solutes that is induced by exposure to low temperatures, prevents cells from excessive loss of water. The decrease in osmotic potential of tissues during cellular dehydration is suggested to result from the accumulation of soluble sugars in cells, although other compounds also contribute to it (Ingram & Bartels 1996). The osmotic potential of needles decreased in cold-treated Scots pine seedlings (II) and during the autumn in mature trees (I). Needle water potential followed the same pattern as osmotic potential in cold-treated seedlings (II). The decrease in osmotic and water potential cannot be totally explained by sugars, because osmotic potential decreased even when sugar concentrations started to decrease in the cold treatment (II).

Osmotic potential increase during deacclimation in conifer seedlings (Wang & Zwiazek 1999), which was also detected in experiment V. A decrease in the water content of mature Scots pine needles has been observed during the spring (Huttunen *et al.* 1981, Havas & Hyvärinen 1990, Sutinen *et al.* 2000) and a significant reduction in osmotic potential occurred in mature Scots pine needles during March-May (I). The difference between the osmotic potentials of mature trees and seedlings may result from the fact that seedlings that overwinter below snow cover have a better availability of water. Furthermore, mature trees can be exposed to sun and frost in spring (Sutinen *et al.* 2000). The lack of snow cover in wintertime causes dehydration stress also in bilberry, which is seen as a lowered osmotic potential in spring compared to plants below the snow (Havas 1971).

Soluble sugars accumulate in woody plants during the natural hardening period in autumn (Pomeroy & Siminovitch 1970, Nozzolillo *et al.* 1990, Fischer & Höll 1991, Oleksyn *et al.* 2000) and their concentrations in conifer needles are positively correlated with frost hardiness (Aronsson *et al.* 1976, Ögren *et al.* 1997, Greer *et al.* 2000). Lowering of sugar content is reported to decrease the frost resistance of spruce and pine (Ögren *et al.* 1997). The increase in sugar concentration may be a result from the degradation of starch (Fischer & Höll 1991), since starch concentration in needles of conifers decreases during cold acclimation (Aronsson *et al.* 1976, Fischer & Höll 1991, Greer *et al.* 2000). In this study, the concentrations of soluble sugars increased at the same time as a decrease in the starch concentration in the needles of the cold-treated seedlings was observed (II).

The soluble sugar concentration of needles started to decline after 10 days in the cold treatment while the starch content of needles began to increase (II). This indicates that the acclimation of Scots pine seedlings to low temperature happened in less than 10 days (II). Since low temperatures cause photoinhibition in Scots pine needles (Krivosheeva *et al.* 1996), the increase in the starch concentration may be a sign of the recovery of photosynthesis in the cold. The accumulation of sugars may also indicate the lack of an active sink in the cold-treated seedlings (Fischer & Höll 1991), because their growth was

repressed. Starch accumulates in conifer needles before budbreak in the spring (Pomeroy *et al.* 1970, Fischer & Höll 1991, Oleksyn *et al.* 2000), when the photosynthetic activity is already high. The accumulated carbohydrate pool declines after the onset of shoot growth (Ericsson 1979, Fischer & Höll 1991, Oleksyn *et al.* 2000). The depletion of soluble sugars and starch in the needles occurred concomitant to the beginning of growth in the pine seedlings grown at the control temperature (18 ºC) (II). The sugar concentration of needles decreased at the same time as the starch concentration, which also supports the assumption that sugars are allocated to the growing shoot.

5.2 Responses to nitrogen fertilization

Nitrogen and protein concentrations in needles increased in the seedlings that were analyzed during the spring (V). However, the summer fertilizations did not induce a rise in protein concentration of needles in autumn (IV), although the nitrogen contents of the seedlings were markedly elevated by the fertilization (IV, Taulavuori 1998). The nitrogen may be translocated to other nitrogenous compounds in the early autumn. A high level of the polyamines putrescine and spermine accumulated in the nitrogen-fertilized seedlings in the same experiment (Taulavuori *et al.* 1999b) and the concentration of amino acids is known to increase in response to nitrogen fertilization in Scots pine (Näsholm & Ericsson 1990). The increased protein content of fertilized needles in spring (V) may be explained by the adequate time to incorporate the nitrogen into proteins. The rise in protein concentration was also seen in the soluble protein patterns, where the amounts of certain components were found to be higher in the N-fertilized seedlings (V). The protein content of buds in the NPK-fertilized trees showed no response to fertilization (III) which may be due to the fact that needles and bark are regarded to be the major nutrient reserve for growth in conifers (Fife & Nambiar 1984, Gezelius 1986).

Soluble protein patterns of both buds (III) and needles (IV, V) showed differences in response to nitrogen fertilization. Some proteins in the needles were more abundant in the nitrogen-fertilized seedlings, which suggests that the proteins could act as a storage form of nitrogen. The abundance of bark storage proteins (Coleman *et al.* 1994), as well as the VSP homologues in the leaves of *Populus* (Lawrence *et al.* 1997), are regulated by nitrogen availability. A decrease in certain protein components was also seen in the buds of fertilized trees in spring, while the amounts of these proteins were unchanged in the unfertilized trees. This observation may indicate an earlier growth start in the fertilized trees (Bobbink *et al.* 1992).

The amount of 60-kDa dehydrin in Scots pine tissues (I, V), which was highest in winter, decreased earlier in the N-fertilized seedlings than in the unfertilized seedlings during deacclimation (V). The level of this dehydrin declined in the fertilized seedlings during the autumn while it was not detected at all in the unfertilized seedlings (IV). Because nitrogen fertilization increases leaf biomass and area of trees (Nilsen 1995, Fife & Nambiar 1996), it may also affect their water use. Fertilization has been shown to increase the consumption of water (Nilsen 1995). Also, an increased sensitivity to drought has been detected in plants that have been receiving an excess of nitrogen (Dueck *et al.* 1998,

Gordon *et al.* 1999). The appearance of the 60-kDa dehydrin in the N-fertilized seedlings may thus reflect the difference in the water status of fertilized and unfertilized plants.

Nitrogen fertilization increases the growth of conifers (Valinger 1992, Hynynen 1995, Fife & Nambiar 1997) and the level of nitrogenous substances in them (Zedler *et al.* 1986, Ferm *et al.* 1990, Näsholm & Ericsson 1990, Taulavuori *et al.* 1999b). The apical shoot growth of the seedlings increased due to nitrogen fertilization but the dose of fertilizer did not affect the degree of growth (V). NPK-fertilization also increased the diameter growth of trees (III).

The impact of nitrogen on frost resistance varies according to species, the amount and the form of nitrogen. The age of the plant has also shown to affect the response. Ammonia fumigation has caused frost sensitivity in the needles of mature Scots pine (Clement 1996), but not in seedlings (Clement *et al.* 1999). The dieback of Scots pine near farms emitting high amounts of ammonia (Ferm *et al.* 1990, Pietilä *et al.* 1991) has been suggested to be the consequence of frost injuries. The Scots pine seedlings frost hardened in autumn (Taulavuori 1998, IV) and deacclimated during the spring (V) in a natural regime. The frost resistance of needles was not affected by nitrogen fertilization in the Scots pine seedlings (V, Taulavuori 1998). Although seedlings fertilized with nitrogen are reported to begin their growth earlier in the spring (Bobbink *et al.* 1992), the earlier growth start of fertilized seedlings that was observed here did not affect the frost resistance of needles (IV). Because frost resistance may vary between different tissues within the plant (Weiser 1970, Sakai & Larcher 1987) and most of the injuries detected in Scots pine occur in the buds, the measurement of needle frost resistance may not be an adequate indicator of the hardiness of this species.

5.3 Future prospects

In this research I have shown for the first time the role of dehydrins in the metabolism of Scots pine. Several dehydrins were found during different experiments. A 60-kDa dehydrin observed in all the tissues studied was related to the development of frost resistance or to the decreasing osmotic potential. The dehydrins of needles in Scots pine seedlings showed no response to low temperature treatment. A possible extension of this study would address the regulation of dehydrin-related proteins and dehydrin gene expression during cold and drought stress and the effect of photoperiod on Scots pine. Also the interesting finding that dehydrin expression between mature and juvenile Scots pine showed a difference warrants further research. Characterization of proteins that respond to the change of season and to nitrogen fertilization with increasing concentration would give more information about the metabolism of Scots pine during frost resistance or dormancy transitions.

The results of nitrogen fertilization experiments on the water balance of Scots pine as well as on dehydrin expression suggested a difference in the water status of fertilized and unfertilized plants. Since nitrogen fertilization did not show an effect on the frost resistance of needles, a more detailed study on the frost resistance of buds and on the water relations of needles and buds would clarify the effects of nitrogen fertilization on Scots pine.

6 Conclusions

The dehydrin proteins in Scots pine tissues fluctuated in response to changes in season and nitrogen fertilization. A 60-kDa dehydrin was found to be dominant in buds and bark of mature trees and in needles of seedlings in the winter. This dehydrin increased in concentration in needles of mature trees in the spring. The increases in concentration were related to the development of frost resistance or to the decreasing osmotic potential, which may indicate that the 60-kDa dehydrin is linked to the resistance to frost and desiccation stresses during the winter. Other dehydrins were also found in the pine tissues but they were seen during the summer or deacclimation. In mature trees, different dehydrins appeared in the buds during the springtime, which may indicate alterations in the water balance of buds as was detected in the needles. Because the low temperature treatment alone did not induce any dehydrins in the needles, the regulation of dehydrins by other factors, such as photoperiod, is possible. However, these results were obtained in the seedlings and their response may differ from that of the mature trees. Therefore the effect of the low temperature on the induction of dehydrins in Scots pine needles cannot be excluded.

Soluble protein concentration exhibited a seasonal pattern in bark and buds of mature trees, as well as in acclimating seedlings. Several specific soluble proteins increased in amount during the winter in Scots pine tissues and some of these were more abundant in the nitrogen-fertilized seedlings. These proteins may serve as storage compounds to support new growth in the spring, because their concentration in tissues decreased at the onset of growth. Other roles of these proteins, e.g. in frost resistance or dormancy transitions of Scots pine, warrants further research.

Soluble sugar and starch concentrations responded to low temperature treatment immediately. Their levels started to change direction again in less than ten days, which may reflect the acclimation of carbohydrate metabolism to low temperature.

Nitrogen fertilization increased the growth and the amount of proteins in Scots pine, but did not affect frost resistance or the osmotic potential of needles in seedlings. However, the results of the soluble protein and dehydrin studies indicate that the earlier growth start of the fertilized plants may have an effect on their water balance and thus affect their tolerance to dehydration related stresses.

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