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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public criticism in Auditorium XIV of the University Main Building, Unioninkatu 34, on 18 May 2002, at 10 o´clock in the morning

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Cover photo: Hydroponically grown Scots pine seedlings exposed to low (light colour) or high (dark colour) level of nutrients (Photographed by Risto Rikala)

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Abstract

Iivonen, S. 2002. Responses of Scots pine (*Pinus sylvestris* L.) seedlings to root zone temperature and nutrient supply in hydroponic culture during the second growing season. The Finnish Forest Research Institute, Research Papers – Metsäntutkimuslaitoksen tiedonantoja 833. 56 p.

In this thesis are analyzed the whole-tree physiological responses of one-year-old Scots pine (*Pinus sylvestris* L.) seedlings to root zone temperature (RZT) and nutrient supply throughout the second growing season. In addition, seasonal root growth and root activity were investigated in relation to shoot phenology and carbohydrate status of the seedlings. The thesis focuses on root and shoot growth, gas exchange of the seedlings, nitrogen net uptake, H^+-ATPase (PM-ATPase) activity and lipid composition of the root plasma membrane in response to different RZT regimes and nutrient supply in spring. Seasonal shoot and root growth, gas exchange of the seedlings, chlorophyll fluorescence of the needles, nitrogen net uptake, contents of starch and soluble sugars in different plant organs, proteins in the root plasma membrane and the amount and activity of root PM-ATPase were studied over the growing season. All the experiments were conducted in hydroponic culture in a growth chamber where RZT, nutrient availability, air temperature, air humidity and light conditions were controlled.

In spring at a RZT of 5°C, and to some extent also at temperatures of 9–12°C, net photosynthesis, transpiration and stomatal conductance were limited. The benefit to net photosynthesis provided by high nutrient supply became evident when RZT of 9°C was reached, which might be explained by changes occurring in plasma membrane lipid composition of the roots. The increase in phospholipid fatty acid unsaturation and the increase in sitosterol/stigmasterol ratio of root plasma membranes after long-term exposure to low RZTs might be a coping mechanism developed to limit the negative effects of low RZT under conditions of high nutrient supply.

Root growth was clearly dependent on RZT in spring, being strongly restricted at RZT of 5°C and limited at RZTs below 13°C. The real PM-ATPase activity of the roots was also low until RZT was increased to 9°C in spring. A decrease in the phospholipid content, increase in the phospholipid fatty acid saturation, decrease in the phospholipid/free sterol ratio and changes in free sterol composition occurred at RZTs that allowed root growth. This suggests that these changes in plasma membrane lipid composition are used to adjust the function of the plasma membrane in root cells to increasing temperatures. The hydrolytic activity of root PM-ATPase was, however, not regulated by the bulk lipid composition of the plasma membrane. Shoot growth was not as sensitive to RZT as root growth was, but at a constant RZT of 5°C it was limited and when the RZT increased slowly in spring it was delayed. Thus, shoot growth is determined both by air temperature and by RZT in spring. High nutrient supply promoted growth of the roots and shoot only after the RZT was increased to 13°C.

The results of this study show that seasonal growth of the stem, needles and roots of one-year-old Scots pine seedlings occurs in phases that lead to episodic growth of the shoot and roots. Structural growth of the roots as well as net accumulation of soluble sugars and starch in roots was most intense at the end of the growing season after the period of intensive needle elongation and after most of

the shoot growth had taken place. This confirms the idea that root growth is slowed down during the time of intensive shoot growth because the growing shoot is a strong consumer of photoassimilates. The nutrient supply did not significantly affect seasonal root growth, but the total growth of the roots was favoured by high nutrient supply. Rapid root growth led to an increase in the total PM-ATPase activity of root system because PM-ATPase activity of the current roots was much higher than the activity of the previous-year roots due to the larger amount of enzyme in the current roots. The results of this thesis also show that the response of root growth and PM-ATPase activity to RZT did not remain in the same throughout the growing season; consequently, RZTs that limit root growth and activity in spring can be favourable in autumn.

These results provide new information on seasonal root growth and whole-tree physiological responses of one-year-old Scots pine seedlings to RZT and nutrient supply in spring and throughout the growing season. This information can be utilized in reforestation practises to take advantage of periods when root growth and activity in seedlings are high enough to promote their rapid establishment on planting sites.

Keywords: chlorophyll fluorescence, free sterols, gas exchange, growth, H^+-ATPase, nitrogen, phospholipids, plasma membrane, roots, shoot, soluble sugars, starch

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Preface

This study was carried out at Suonenjoki Research Station of the Finnish Forest Research Institute during the years 1996–2000. I am grateful for the excellent working facilities provided by Suonenjoki Research Station. The study was supported by the Maj and Tor Nessling Foundation, the Metsämiesten Säätiö Foundation, the Society of Finnish Forest Research, the Finnish Cultural Foundation, the Niemi Säätiö Foundation and the Graduate School of Forest Sciences, all of which are gratefully acknowledged.

I wish to express my sincere thanks to numerous people who have, in one way or another, contributed to this work. My warmest thanks to Dr. Elina Vapaavuori for the supervising my work and for her trust in my skills to do scientific research independently. I thank Dr. Aija Ryyppö and Dr. Risto Rikala warmly for collaboration and valuable advice throughout the course of this work. I am grateful to Dr. Marja-Liisa Sutinen for her help with analysis of fatty acids and to Dr. Pekka Saranpää for his help with analysis of free sterols. I also thank Dr. Juha Lappi and Dr. Risto Häkkinen for statistical guidance. I thank Professor Pasi Puttonen for his valuable comments on the manuscript. Professor Marjatta Raudaskoski and Professor Victor Lieffers are acknowledged for their careful work in reviewing the manuscript and Dr. Joann von Weissenberg for revising the English language of this thesis. I would like to express my gratitude to Ms. Mervi Ahonpää, Ms. Marja-Leena Jalkanen, Mr. Jukka Laitinen, Mr. Tapio Laakso, Ms. Maija Piitulainen, Ms. Hanna Ruhanen, Ms. Eeva Vehviläinen, Mr. Pekka Voipio and Ms. Anna-Maija Väänänen for their excellent laboratory and technical assistance. I also thank the family of Helena and Martti Vuorinen for their hospitality during my stay in Suonenjoki.

Finally, I extend my warmest thanks to my husband Markku and my children Emma and Akseli for their love, which has made the recent years of my life so happy.

Rauma, December 2001 *Sari Iivonen*

Contents

List of original articles

This thesis is based mainly on the following articles, which are referred to in the text by their Roman numerals. Additional data are also presented.

I **Ryyppö, A., Iivonen, S., Rikala, R., Sutinen, M.-L. & Vapaavuori, E.** 1998. Responses of Scots pine seedlings to low root zone temperature in spring. Physiologia Plantarum 102: 503–512.

II **Iivonen, S., Rikala, R., Ryyppö, A. & Vapaavuori, E.** 1999. Responses of Scots pine (*Pinus sylvestris*) seedlings grown in different nutrient regimes to changing root zone temperature in spring. Tree Physiology 19: 951–958.

III **Iivonen, S., Rikala, R. & Vapaavuori, E.** 2001. Seasonal root growth of Scots pine seedlings in relation to shoot phenology, carbohydrate status and nutrient supply. Canadian Journal of Forest Research 31: 1569–1578.

IV **Iivonen, S. & Vapaavuori, E.** 2002. Seasonal variation in nitrogen net uptake and root plasma membrane H^+-ATPase activity of Scots pine seedlings as affected by nutrient availability. Tree Physiology 22: 1–10.

In Study I S. Iivonen isolated plasma membranes, measured purity of plasma membrane preparations, measured PM-ATPase activities and analysed these results. She participated in the analysis of growth and gas exchange measurements as well as writing of the article. In Studies II-IV S. Iivonen did most of the planning of the experiments and was responsible for the analyses and writing the articles.

List of abbreviations

ADP	adenosinediphosphate
AMP	adenosinemonophosphate
ATP	adenosinetriphosphate
CTP	cytidinetriphosphate
d.d.	degree days above a threshold 5°C
F_v/F_m	ratio of variable to maximal chlorophyll fluorescence
FW	fast warming
GC	gas chromatography
GTP	guanosinetriphosphate
HN	high nutrient
IDP	inosinediphosphate
ITP	inosinetriphosphate
kD	kilodalton
LN	low nutrient
MS	mass spectrometry
NADH	nicotinamide adenine dinucleotide (reduced form)
P_i	inorganic orthophosphate
PC	phosphatidylcholine
PE	phosphatidylethaloamine
PM	plasma membrane
PM-ATPase	plasma membrane H^+-ATPase
PS	phosphatidylserine
PSII	photosystem II
RGR	relative growth rate
RZT	root zone temperature
SE	standard error
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SW	slow warming
UTP	uridinetriphosphate

1 Background of the study

About 60 million Scots pine (*Pinus sylvestris*) seedlings are outplanted in Finland each year (Västilä and Herrala-Ylinen 2000). The main target of reforestation by planting is to obtain rapid establishment of the seedlings at the planting site and to raise these planted seedlings to stems of commercial value. In practise, however, in Finland and Sweden the mortality of planted Scots pine seedlings during the 5 to 10 years after planting has varied from 15% to 60% (Karjula et al. 1982, Elfving 1992, Saksa 1998). The most common reasons for death of planted seedlings are insects, ground vegetation and planting mistakes; but in almost every third case, the cause of death could not be identified (Saksa 1998). One of these unidentified reasons may be planting stress caused by low soil temperature during planting since conifer seedlings are usually planted in spring prior to flushing, when soil temperature is below 10°C. In southern Finland, the forest soil temperature is below 10°C until late May (Yli-Vakkuri 1960), and in northern Finland soil temperature increases even more slowly (Kubin and Kemppainen 1991).

Low soil temperature inhibits root growth, thus preventing the formation of root-soil contact, which is necessary for water and nutrient uptake of the seedling (Burdett 1991). Because air temperature can be much higher than soil temperature, especially on sunny days, shoot growth is promoted. The difference in air and soil temperature may lead to imbalance between transpiration and water uptake, thereby inducing water deficit in the elongating shoot (Lopushinsky and Kauffman 1984), which can impair the establishment of seedlings at the planting site. In addition to environmental factors, genetic regulation also determine the seasonal distribution of biomass among the organs of the seedling; and therefore the rate of root growth may vary during the growing season due to internal factors. In Scots pine, the seasonal changes in growth of the above-ground organs are well-studied (Mikola 1950, Leikola 1969, Raulo and Leikola 1974, Parviainen 1975, Junttila and Heide 1981), while less information is available about root growth. Seasonal changes in the activity of the root system in terms of nutrient uptake are poorly known. In general, the activity of roots in nutrient uptake is higher in new unsuberized roots than in older suberized roots (Chung and Kramer 1975, Jensén and Pettersson 1980). During the growth process, the most active part of the root system is continuously renewed, and the activity of the older parts of the root system decreases. Due to this dynamic

behaviour of the root system, its activity may vary considerably during the growing season. Better knowledge of the seasonal pattern of root growth and activity as well as better knowledge of factors that affect them can serve to improve reforestation practises and to maximize establishment of Scots pine seedlings at planting sites.

2 Literature review

2.1 Phenological development of Scots pine during the growing season

2.1.1 Shoot development

In boreal forests, growth of conifers includes marked seasonal changes that are very important for their survival in extreme climatic conditions. The annual growing cycle of Scots pine is divided into the active period and dormancy phases (Sarvas 1972, 1974). The growth and development of seedlings during the active period is regulated by internal and environmental factors. Of the environmental factors, the importance of air temperature and daylength is pronounced, but soil moisture, soil temperature and nutrient availability also affect the development of seedlings.

Bud burst occurs in spring after chilling requirements have been met and the bud has been exposed to temperatures above a certain threshold for a sufficient time. Bud burst occurs even in complete darkness, which means that light does not influence timing during the early phase of the active period (Koski 1990). Bud burst is followed by a phase of shoot extension and new bud formation. In pines, the shoot grows from a pre-formed bud (Lanner 1967); and in Scots pine, shoot elongation starts after terminal bud break when the air temperature sum is about 10 d.d. (Raulo and Leikola 1974). Scots pine is among those species that exhibit pre-determined growth, which means that the number of stem units in a bud is determined by the weather conditions of the previous year (Mikola 1950, Junttila and Heide 1981); but the rate of shoot elongation and length of the internodes between needles are influenced by the growing conditions of the current season (Garret and Zahner 1973, Raulo and Leikola 1974, Junttila 1986). A long photoperiod is needed for needle elongation of Scots pine (Wareing 1950, Ekberg et al. 1979). In summertime, the light requirement is satisfied at high latitudes; therefore, at this time needle elongation is strongly dependent on air temperature (Junttila and Nilsen 1993). Needle elongation occurs after considerable shoot elongation (Dougherty et al. 1994) and continues after the shoot has completed its elongation and the terminal bud has been formed (Parviainen 1975, Rikala and Huurinainen 1990). Diameter growth of the stem can remain high throughout the season if environmental conditions are favourable, and thus diameter growth can continue after formation of the terminal bud (Leikola 1969, Dougherty et al.

1994). Decreasing daylength appears to be an important factor in initiating the rest period of needles and stem (Smit-Spinks et al. 1985, Bigras and D'Aoust 1993). After terminal bud set, the shortening daylength initiates the development of needle frost hardiness, which can also be improved in the final stages of needle growth (Hurme et al. 1997, Rikala and Repo 1997). Hardening of the stem begins later than hardening of the needles (Ryyppö 1998). Also in Norway spruce (*Picea abies*), the critical daylength for cambial growth is shorter than that for apical growth and cambial growth ceases several weeks later than apical growth does (Heide 1974).

2.1.2 Root development

In contrast to the shoot, root growth is not affected by photoperiod (Heide 1974, Hawkins et al. 1996) and roots do not have an internally controlled period of dormancy. Therefore root growth can occur throughout the season if other environmental conditions permit (Lyr and Hoffman 1967, Dewar et al. 1994). At northern latitudes, low soil temperatures usually inhibit root growth in wintertime and root growth is resumed in spring when soil temperature permits (Lyr and Hoffman 1967). The phenology of root development is not well understood. Studies of seasonal variation in the living root biomass of Scots pine stands have reported considerable variation during the growing season but no distinct and clear pattern (Persson 1978, 1979, Makkonen and Helmisaari 1998). It has been suggested that this temporal variation is largely determined by changes in environmental conditions (Persson 1983). The low soil moisture in summer and low soil temperature in spring and autumn are thought to be the most important environmental factors limiting root growth (Dougherty et al. 1994, Eissenstat and Van Rees 1994). However, episodic shoot and root growth has been reported in seedlings of several pine species such as *Pinus sylvestris* (Lyr and Hoffman 1967), *Pinus contorta* (Cannel and Willet 1976) and *Pinus resinosa* (Drew 1982). The most common explanation for this phenomenon has been competition for carbohydrates among the different organs (Ritchie and Dunlap 1980, Deans and Ford 1986, Eissenstat and van Rees 1994). In field conditions, large variation in environmental conditions as well as methodological difficulties involved in root sampling are probably the main reasons for difficulties to distinguish between the importance of shoot phenology and soil moisture as factors affecting root growth. Furthermore, especially in field studies, the above- and below-ground phenology of Scots pine are seldom considered simultaneously (Persson 1978, 1979, Makkonen and Helmisaari 1998).

2.2 Role of stored and currently formed photosynthates in seasonal development of conifers

Plant growth can be facilitated by currently assimilated carbon or by carbon mobilized from previously stored reserves. In conifer seedlings, growth is supplied mainly by carbon fixed that year (Dewar et al. 1994, Lippu 1999); but when the consumption of current photosynthates exceeds the production, previously formed reserves can be utilized (van den Driessche 1987). In regions where the winters are cold, photosynthesis of conifers is usually depressed during the winter months and it recovers in spring (see Öquist and Martin 1986). The recovery of the CO_2 uptake rate is closely related to temperature (Pelkonen and Hari 1980) and in spring and early summer full recovery of the photosynthetic capacity of one-year-old Scots pine shoots can take more than three months (Troeng and Linder 1982). After recovery, the photosynthetic capacity is affected by environmental factors (Man and Lieffers 1997) and the stage of foliage development (Troeng and Linder 1982, Vapaavuori et al. 1995). The major sinks for assimilated carbon in Scots pine vary during the growing season because the distribution of carbon is governed mainly by the sink strength of different organs (Lippu 1999). Because the strength of a particular sink depends on its size and metabolic activity (Ho 1988), environmental conditions affect sink strength.

2.3 Responses of conifer seedlings to low root zone temperatures in spring

2.3.1 Root and shoot growth

Growth is determined by cell division, cell expansion and mass deposition of the cell material (see Lambers et al. 1998). After the root cell has divided, it elongates, provided that the turgor pressure exceeds a certain threshold value and necessary changes occur in the mechanical properties of the cell-wall (Lambers et al. 1998). Changes in the mechanical properties of the cell-wall, rather than changes in turgor, are responsible for control of the elongation rate of roots (Pritchard 1994). Low root zone temperature (RZT) limits the root growth of conifer seedlings in spring (Grossnickle and Blake 1985, Lopushinsky and Max 1990, Vapaavuori et al. 1992, Lyr and Garbe 1995). The low rate of root elongation at low RZTs can be due to several factors and their interactions. Low RZT reduces the metabolic activity and sink strength of the roots, which reduces translocation of carbohydrates to roots (Hurewitz and Janes 1983). Further-

more, low temperature *per se* can decrease the activity of enzymes involved in cell wall extension. In the sunflower, inhibition of root growth at low RZT is associated with reduced rate of cell extension in the root meristems (Burholt and Van't Hof 1971).

Impaired shoot growth by low RZT has been demonstrated for pine seedlings (Lopushinsky and Max 1990, Vapaavuori et al. 1992, Ryyppö et al. 1994). It has been suggested that the main cause of reduced shoot growth at low RZT is hormonal, probably reduced cytokinin supply by roots (Lyr and Garbe 1994, Lyr 1996). Torrey (1976) has shown that environmental factors which interfere with root function reduce the cytokinin content of the xylem exudate. Depression of the production and export of cytokinin due to low RZT has been reported in maize roots (Atkin et al. 1973).

2.3.2 Water and nutrient uptake

Low RZT reduces water absorption by increasing the viscosity of water and lowering the permeability of the membrane to water (Kaufmann 1975, 1977, Markhart et al. 1979). In conifers, new unsuberized roots are more effective in water and nutrient uptake than older roots (Chung and Kramer 1975, Häussling et al. 1988). Therefore, inhibition of root growth at low RZT also reduces water and nutrient uptake. In addition to water movement across the lipid bilayer, symplastic water transport can also occur via water channel proteins, aquaporins (Steudle and Peterson 1998, Kjellbom et al. 1999, Wan and Zwiazek 1999). Plasma membrane aquaporins might offer a low-resistance pathway for water flow through cells and may thus provide a more controlled pathway for water movement than would be possible if the principal pathway were via the lipid bilayer (Maurel and Chrispeels 2001).

Low temperature may affect the structure and function of transport enzymes in the plasma membrane directly (Hällgren and Öquist 1990). The decrease of RZT by 10°C in the temperature range of 5–25°C reduced the net uptake of N, K and P by 50% in Scots pine roots (Jensén and Pettersson 1980). However, over a longer term, the degree to which RZT alters nutrient uptake capacity is determined by the degree to which growth and the root:shoot ratio are affected (BassiriRad 2000). When air temperature allows shoot growth, in several herbaceous plants nutrient uptake has been shown to increase after long-term exposure to low RZT (White et al. 1987, 1991, Engels et al. 1992). This adjustment of nutrient uptake to growth demand may mean that, despite lower root growth, shoot growth is not greatly limited by low RZT when a plant grows in nutrient-rich substrate (Marschner 1995).

2.3.3 Gas exchange

Reduced rates of gas exchange with low RZT have been shown in several conifers (DeLucia and Smith 1987, Day et al. 1990, 1991, Vapaavuori et al. 1992). At low RZTs, the water potential of the shoot often decreases, which leads to a decrease of stomatal conductance and therefore stomatal limitation of photosynthesis (Day et al. 1991). Other mechanisms may also be responsible for lower rates of gas exchange in cold soils. Stomatal limitation of photosynthesis has been demonstrated in conifers without changes in bulk water potential of the shoot (Day et al. 1989, 1990), which may indicate that stomata respond to non-hydraulic signals from roots that are experiencing drought (Dodd et al. 1996) or low temperature (Chen et al 1983, Lee et al. 1993). Non-stomatal limitation of photosynthesis by low RZT has also been found in conifers (DeLucia 1986) such as starch accumulation to needles and stem (DeLucia 1986), which may cause a feedback inhibition of photosynthesis (Herold 1980).

2.4 Effect of nutrient supply on growth and gas exchange

Nitrogen is the major growth-limiting mineral nutrient of higher plants (Marchner 1995) and typical responses to nitrogen shortage are decreased gas exchange (Evans 1996, Walcroft et al. 1997), decreased total biomass production (Coutts and Philipson 1976, Friend et al. 1990) and increased root:shoot ratio (Ingestad and Kähr 1985, Ericsson 1995). Both light and dark reactions of photosynthesis are strongly affected by nitrogen deficiency. Decreased rate of electron transport and carboxylation (Walcroft et al. 1997) have been demonstrated under conditions of nutrient deficiency, which might be due to decreased amount and activity of RuBP carboxylase and reduced concentration of chlorophyll (Gezelius 1986). Furthermore, nutrient deprivation has been shown to lead to stomatal closure and reduced transpiration (Chapin el al. 1988) as well as to reduced hydraulic conductance of roots (Chapin et al. 1988, Radin and Matthews 1989) and decreased water potential in shoots (Paquin et al. 2000).

2.5 The root plasma membrane

2.5.1 *Structural components of the plant plasma membrane*

The plasma membrane (PM) forms the outermost boundary of the living cell. It functions as an active interface between the cell and its environment by controlling the transport of molecules into and out of the cell and by transmitting signals from the environment to the cell. Plant PMs consist of lipids, proteins and carbohydrates in the molecular ratio of about 40:40:20 (Larsson et al. 1990). The main lipid classes in plant PMs are phospholipids and free sterols, which exist in proportions that vary between species and tissues (Yoshida and Uemura 1986, Rochester 1987). In several species, the most common phospholipids of root PMs are phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Gronewald et al. 1982, Whitman and Travis 1985, Norberg and Liljenberg 1991); and the most abundant free sterols are sitosterol, campesterol and stigmasterol (Rochester et al. 1987, Burgos and Donaire 1996). Phospholipids contain a polar head group and two hydrophobic fatty acid tails. The fatty acid tails contain 14–24 carbon atoms and can have one or more double bonds. The most common fatty acids of the phospholipids in the PMs are palmitic acid (C16:0), linoleic acid (C18:2) and linolenic acid (C18:3) (Larsson et al. 1990). The kinks introduced by double bonds affect the conformation of the fatty acid tail and therefore the packing of the molecules in the lipid bilayer, which, in turn, affects the fluidity of the membrane. Membrane sterols are thought to serve as membrane fluidity 'buffers'. They increase membrane fluidity at low temperatures by disrupting the gelling of phospholipids and decrease fluidity at high temperatures by interfering with the flexing motions of the fatty acid tails (Schuler et al. 1990, 1991, Staehelin and Newcomb 2000).

2.5.2 *Changes in lipid composition of the plasma membrane during cold acclimation and deacclimation*

In the boreal zone, forest trees are exposed to great variation in temperature during their annual growing cycle. To be able to avoid freezing injuries in winter, the lipid structure of the PM has to undergo structural changes to maintain optimum membrane fluidity by a process known as cold acclimation (Levitt 1980, Thompson 1984, Lynch and Steponkus 1987). However, not all changes in lipid composition during cold acclimation are necessarily related to development of frost hardiness *per se*, but may rather be needed for metabolic adjustment to low temperatures (Palta et al. 1993). In overwintering

plants, these changes in lipid composition are reversed during cold deacclimation, when cells have to maintain optimum fluidity in the face of increasing temperature (Yoshida 1986, Sutinen 1992). During cold deacclimation, in extremely cold-hardy trees, a decrease in phospholipid content, a decrease in degree of unsaturation in phospholipid fatty acids, a decrease in phospholipid/sterol ratio as well as a decrease in PM fluidity have been observed (Yoshida 1986, Sutinen 1992). There is no information available on changes in the sterol composition of the PM of conifers in response to cold acclimation or deacclimation. In herbaceous species, an increase in free sterols (Lynch and Steponkus 1987, Palta et al. 1993) or no change in free sterols (Uemura and Yoshida 1984) has been found during cold acclimation. Substantial changes also occur in the molecular composition of free sterols during cold acclimation of herbaceous species; e.g. the proportion of sitosterols increases gradually with a corresponding decrease in campesterols and stigmasterols (Uemura and Yoshida 1984, Lynch and Steponkus 1987, Palta et al. 1993).

2.5.3 *Structure and function of the plasma membrane H^+-ATPase*

H^+-ATPases are enzymes that generate or retrieve the energy stored in H^+ gradients by coupling ATP hydrolysis/synthesis to proton flow across membranes (see Palmgren 1998). There are three classes of H^+-ATPases in plant cells: 1. F-type ATPases functioning as ATP synthases in the inner mitochondrial membrane, 2. V-type ATPases in the tonoplast and 3. P-type ATPases in the PM. The plasma membrane H^+-ATPase (PM-ATPase; EC 3.6.1.35) is an integral membrane protein about 100 kD in size that is composed of a single subunit with 10 transmembrane domains (Wach et al. 1992). PM-ATPase hydrolyzes ATP to generate proton transport outside the PM thus producing an electrogenic proton gradient across the PM (Serrano 1990, Michelet and Boutry 1995). In the roots, PM-ATPase is involved in several physiological functions: it provides energy for nutrient uptake, xylem loading and phloem unloading of solutes, and it is involved in regulation of intracellular and extracellular pH and cell turgor (Palmgren 1998). The involvement of PM-ATPase in cell elongation is based on its role in extracellular regulation of pH. According to the 'acid-growth' theory, activation of PM-ATPase leads to acidification of the cell wall, which in turn promotes wall loosening via clevage of load-bearing bonds in the cell wall or activation of lytic enzymes, thus allowing turgor-driven expansion of the cell (Rayle and Cleland 1992).

In *Arabidopsis*, at least 10 genes (AHA1–10) are known to encode the distinct PM-ATPase isoforms (Sussman 1994). Three major iso-

forms, AHA1, AHA2 and AHA3, differ with respect to their kinetic properties (Palmgren and Christensen 1994) and their expression in different tissues (Michelet and Boutry 1995, Palmgren 1998). Studies with oat roots indicates that the concentration of PM-ATPase in PM is fairly low: PM-ATPase represents less than 1% of the PM proteins (Sussman 1994). The abundance of PM-ATPase varies between tissues and cell types. In roots, PM-ATPase is enriched particularly in the root cap, epidermis and endodermis as well as in the companion cells of the phloem (Parets-Soler et al. 1990, Jahn et al. 1998).

2.5.4 Regulation of PM-ATPase at different levels

PM-ATPase has been shown to be regulated at the transcriptional, translational and post-translational levels (Palmgren 1998). The expression of root PM-ATPase is regulated by developmental stage and environmental conditions (Roldán et al. 1991, Michelet et al. 1994, Ahn et al. 1999). The translational regulation may affect, in particular, the expression of certain isoforms, which may result in rapid modulation of PM-ATPase synthesis in response to environmental signals (Michelet et al. 1994, Michelet and Boutry 1995). At the post-translational level, the amount of PM-ATPase can be regulated by the factors which affect the rate of enzyme transport to the PM and the protein turnover rate in the PM. For example, the growth hormone auxin is known to induce membrane flow from the endoplasmic reticulum to the PM, and therefore the increased amount of PM-ATPase in response to auxin treatment can be related to enhanced rate of exocytosis (Hager et al. 1991).

There are several external and internal factors which can cause regulation of the activity of the pre-existing PM-ATPase. However, the mechanism of regulation has been elucidated in only a few cases, and in some cases it is not even clear whether the observed changes reflect modulation of the amount or the activity of the enzyme. The C-terminal regulatory domain of the PM-ATPase constitutes a target for regulatory molecules. It has recently been demonstrated that PM-ATPase is activated by the binding of 14-3-3 protein to the C-terminal region of the enzyme (Baunsgaard et al. 1998, Svennelid et al. 1999). The binding of 14-3-3 protein to PM-ATPase is facilitated by the phosphorylation of Thr-948 in the C-terminal region (Olsson et al. 1998, Svennelid et al. 1999). Due to contradictory results, the role of auxin in activation of pre-existing PM-ATPase still remains questionable (Gabathuler and Cleland 1985, Santoni et al. 1991, Cho and Hong 1995, Jahn et al. 1996). The synthetic cytokinin, benzyladenine, seems to activate the functioning of root PM-ATPase (Kuiper et al. 1991). A positive correlation has been demonstrated

between PM-ATPase activity and the mineral nutrient level to which roots are exposed (Kuiper et al. 1991). However, the effect of nutrient availability on PM-ATPase activity is not consistent, and increased PM-ATPase activity with decreased mineral nutrient supply has also been reported (Lindberg and Yahya 1994).

As an integral membrane protein, the activity of PM-ATPase can be regulated by the membrane lipids. Two theories have been presented to account for lipid modulation of PM-ATPase activity (Cooke and Burden 1990). According to the first theory, changes in lipid composition might affect the physical properties of the bulk lipid bilayer, thereby indirectly affecting the ability of the enzyme to undergo conformational changes, and thus resulting in changes in its activity. The second theory is based on direct lipid-protein interaction. In several plant species and tissues there is evidence for sterol (Cooke et al. 1993, Burgos and Donaire 1996, Grandmougin-Ferjani et al. 1997) and phospholipid (Palmgren and Sommarin 1989, Kasamo 1990, Kasamo and Sakakibara 1995) modulation of PM-ATPase activity. Sterols seem to modulate ATPase activity by more than one mechanism and have been suggested to preserve the configuration of the enzyme and help modulate its interaction between phospholipids (Sandström and Cleland 1989). Grandmougin-Ferjani et al. (1997) found that stigmasterol, in particular, stimulates PM-ATPase in corn roots. PM-ATPase requires phospholipids for its activation (Kasamo and Nouchi 1987, Kasamo 1990). PC, PS and lyso-PC seem to be effective stimulators of PM-ATPase activity (Kasamo and Nouchi 1987, Palmgren and Sommarin 1989, Kasamo 1990). Activation is also dependent on the degree of unsaturation of the fatty acyl chains and their length in phospholipids. Activity was shown to decrease with an increase in the length of the fatty acid chain from 16:0 to 18:0 and with an increase in the degree of unsaturation in the order 18:1 > 18:2 > 18:3 (Kasamo 1990).

3 Aims of this study

The first aim of this study was to investigate whole-tree physiological mechanisms by which Scots pine seedlings respond to low RZTs and nutrient supply during bud burst and shoot elongation in spring. The second aim was to determine the seasonal pattern of root growth and root activity in nutrient uptake in relation to shoot phenology, carbohydrate status of the seedlings, RZT and nutrient supply.

The aims were divided into the following specific objectives:

1. To study how different RZTs affect growth, gas exchange, PM-ATPase activity of roots, nitrogen net uptake and lipid composition of the root PM in spring (I, II, additional data).
2. To examine whether nutrient supply affects growth and physiological performance of the seedlings in spring (II, additional data).
3. To examine whether root growth is related to aboveground phenology, and to examine seasonal changes in root growth (III).
4. To examine seasonal changes in the PM-ATPase activity of the roots and nitrogen net uptake of the seedlings and to evaluate the relative role of current- and previous-year roots in the PM-ATPase activity of the root system (IV).
5. To study how nutrient supply affects seasonal growth and PM-ATPase activity of the roots (III, IV).
6. To characterize the temperature sensitivity of root growth and PM-ATPase activity throughout the growing season (III, IV).

4 Materials and methods

An overview of the experimental work is presented here. More detailed descriptions of material and methods can be found in the original articles (I–IV).

4.1 Plant material and growing conditions

The articles of this thesis are based on three experiments. Article I is based on Experiment 1, Article II on Experiment 2 and Articles III and IV on Experiment 3. Lipid composition of root PMs and purity analyses of PM preparations from Experiment 2 (II) are presented as additional data.

The experiments were conducted with one-year-old Scots pine (*Pinus sylvestris* L.) seedlings of the same seed origin. The seeds were collected from an open-pollinated seed orchard in Toivakka, Central Finland (62°05'N, 26°10'E), that was established with material obtained from different sites in Finland between latitudes 61°70 'N and 64°20'N. Seedlings were grown in peat-filled paper pots in the nursery of Suonenjoki Research Station using normal nursery practises. In mid-October, the seedlings were transferred to cold storage and were kept there at –3 to –5°C for four (III, IV), five (I) or six months (II). After cold storage, the seedlings were thawed in darkness at 4–5°C for 7–14 days. Thereafter, the peat surrounding the root system was removed by gentle washing with cold tap water. Immediately after washing, the seedlings were placed into plastic containers filled with aerated tap water (I) or nutrient solution containing a low concentration of nutrients (II, III, IV) and were moved into a growth chamber to recover from the washing procedure. During the first three days the seedlings were kept in continuous dim light (100–125 μmol m^{-2} s^{-1} PAR) and at constant air and root zone temperatures of 5°C (I) or 4°C (II, III, IV) and were thereafter subjected to the experimental conditions.

All the experiments were done in hydroponic cultures in the growth chamber to ensure a constant supply of water and nutrients and to maintain the desired RZTs, air temperatures, air humidity and light conditions. In Experiment 1 (I), the seedlings were grown at a constant RZT of 5, 12 or 20°C and day/night air temperature of 20/15°C for 10 days (Table 1). To maintain a constant nutrient concentration in plant tissues, nutrients were given in relation to the growth rate of the seedlings (Ingestad and Lund 1986). In Experiment 1, the number

Table 1. Description of the growing conditions in the three experiments.

Experiment (Article)	Duration days	Root zone temperature	Air temperature	Photoperiod	Nutrient availability
1 (I)	10	Constant 5, 12 or 20 °C	20/15°C (day/night)	18 h	Nutrients given in relation to growth rate
2 (II)	42	Slow warming: 4 →18°C in 42 days Fast warming: 4 → 18 °C in 22 days and following 20 days maintained at 18 °C	Mean daily air temperature increased from 10 to 18°C in 10 days; then at 18°C for following 32 days	20 h	Constant supply of 0.5 or 3 mM N (other nutrients in proportion to N)
3 (III–IV)	144	Simulated growing season: 5°C → 16.5°C → 6°C	Daily and seasonal variations were simulated	Seasonal variation	Constant supply of 0.25 or 2.5 mM N (other nutrients in proportion to N)

of treatments was three (RZTs as factors). In each treatment were a total of 376 seedlings, and the total number of the seedlings used in the experiment was 1128.

In Experiment 2 (II), during the 42-day period, to simulate a spring of deep frost or no frost in the soil, the increase in RZT was slow (SW-treatment) or fast (FW-treatment), respectively. Air temperature fluctuated similarly in both RZT treatments and the seedlings were further divided into low (0.5 mM N) or high (3 mM N) nutrient treatment (other nutrients were supplied in proportion to N). The nutrient solution used in all the experiments was modified Ingestad's nutrient solution (Ingestad 1979, Ingestad and Lund 1986), which contained major nutrients in weight proportions of 100N : 60K : 18P : 6Ca : 6Mg : 9S. The number of treatments was four (2 RZT treatments × 2 nutrient treatments). In each treatment were 304 seedlings, and the total number of seedlings used in Experiment 2 was 1344.

In Experiment 3 (III, IV), the seedlings were grown over the whole simulated 'growing season'. The climatic conditions simulated the mean temperature, photoperiod and air humidity conditions from May to mid-October in southern Finland (Finnish Meteorological Institute 1991). Seedlings were subjected to low (0.25 mM N) or high (2.5 mM N) nutrient treatment (other nutrients were supplied in proportion to N as in Experiment 2). In both nutrient treatments there were 576 seedlings, and the total number of seedlings used in Experiment 3 was 1152.

4.2 Measurements of biomass and growth parameters

Root volume was determined by a displacement method (Burdett 1979) (I, II) as repeated measurements from the same seedlings. Measured root volume was assumed to equal the fresh mass of roots in hydroponic culture, and thus, shoot fresh mass was determined by subtracting root fresh mass from the mass of the whole seedling (I, II). The fresh mass of the seedling and plant parts were determined by weighing randomly sampled seedlings (III, IV), and the dry mass was determined from subsamples that had been dried at 60°C for 48 h (III). The length of the stem and current needles were measured repeatedly from the same seedling and needle, respectively (III).

4.3 Measurements of gas exchange and chlorophyll fluorescence

Gas exchange of the whole shoot was determined as repeated measurements with a closed-system infrared gas analyser (Li-Cor 6200, Li-Cor, Lincoln, NE, USA) at saturating photon flux density (PAR 550–700 μmol m^{-2} s^{-1}) at 22°C. During the gas exchange measurements, seedlings were kept at the same RZT and nutrient solution as in the treatment. Gas exchange rates were calculated on the basis of silhouette areas of the shoots, which were measured after each gas exchange measurements with a video camera connected to a graphic monitor. The fluorescence induction was measured with a MINI-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany) after 20 min. dark adaption of the needles at room temperature using a 1 s saturating pulse of 9000 μmol m^{-2} s^{-1} (III).

4.4 Analysis of nitrogen, starch and soluble sugars

Total nitrogen concentration of the oven-dried (at 60°C for 48 h) needle and stem samples was measured with a CHN-600 Analyzer (Leco Co., St. Joseph, MI, USA) (II, IV) and that of the root samples with a CHN-900 Analyzer (Leco Co., St. Joseph, MI, USA) (IV). Soluble sugars and starch were analysed from oven-dried plant parts as described by Hansen and Møller (1975) (III).

4.5 Analyses of plasma membranes

Root PMs were isolated by an aqueous polymer two-phase partition (Widell 1987) (I, II, IV). The purity of PM preparations were determined after isolation by measuring the activities of marker enzymes, the substrate specificity and the inhibitor sensitivity (Mattheis and Ketchie 1990) of the PM preparation (I, additional data). Cytochrome *c* oxidase (EC 1.9.3.1) was used as a marker enzyme for the inner mitochondrial membrane (Hodges and Leonard 1974), antimycin A-insensitive NADH cytochrome *c* reductase (EC 1.6.2.4) for the outer mitochondrial membrane and the endoplasmic reticulum (Hodges and Leonard 1974), NO_3^--sensitive ATPase (EC 3.6.1.3) for the tonoplast (Briskin et al. 1987) and Triton X-100 stimulated UDPase (EC 3.6.1.3) for the Golgi apparatus (Nagahashi and Kane 1982). The substrate specificity of the PM fraction was tested for ATP, ADP, AMP, CTP, GTP, IDP, ITP, UDP and UTP. Inhibitors for non-specific phosphatases, mitochondrial ATPase, tonoplast ATPase and PM-ATPase were used in measurements of inhibitor sensitivity.

Activity of PM-ATPase (EC 3.6.1.3) (I, II, IV) was measured at pH 6.7 as described by Ryyppö et al. (1994), with slight modifications, as the amount of Pi released from ATP hydrolysis (Hodges and Leonard 1974). Activity of PM-ATPase was measured at actual RZT and at 38°C and calculated on the basis of PM proteins (I, II) or fresh mass of the roots (IV). The PM-ATPase activity calculated on the basis of total fresh mass of the roots is referred to as 'total activity'. The activity measured at actual RZT is referred to as 'the real PM-ATPase activity' and the activity measured at 38°C is referred to as 'the potential PM-ATPase activity'. The amount of PM protein was estimated according to the method of Bradford (1976). The amount of PM-ATPase was determined by Western blotting using the antiserum raised against the central domain of *Arabidopsis thaliana* (Pardo and Serrano 1989). The PM proteins were first run in SDS-PAGE (Laemmli 1970); thereafter they were transferred electrophoretically to a nitrocellulose membrane, incubated in primary and secondary antibodies and detected with chemiluminisence reagents.

Total lipids were extracted from PM samples by the method of Bligh and Dyer (1959) as described in Palta et al. (1993) (I, additional data). The amount of phospholipids was determined by measuring the content of phosphorus according to the method of Fiske and Subbarow (1925) and calculated assuming an average molecular weight of 769 and phosphorus concentration of 4.03% for phospholipids (Sailerova & Zwiazek 1993) (additional data). For the fatty acid analysis, phospholipids were converted to methyl esters of fatty acids as described in Ryyppö et al. (1994) and separated by GC (I, additional data). The bond index of the phospholipid fatty acids

was calculated as the sum of the product (mol%) of a given fatty acid × number of double bonds (White et al. 1990) according to the equation:

$$\text{Bond index} = C16{:}1 + C18{:}1 + 2 \times C18{:}2 + 3 \times C18{:}3$$

Free sterols in isolated PMs were analyzed by GC-MS after conversion to trimethylsilyl derivatives (additional data). The neutral fraction containing free sterols was dried under gaseous nitrogen, and trimethylsilyl derivatives of free sterols were prepared by adding 0.4 ml 21% (v/v) 1-trimethylsilyl-imidazole (Sigma Chemical Co.) in pyridine. Samples were incubated overnight at room temperature. Cholesterol was used as the internal standard. Sterols were analyzed using HP-6890 GC (Hewlett Packard, Germany) equipped with a mass-selective detector (Hewlett Packard 5873, 70 eV, USA). The trimethylsilyl ethers of sterols were separated on a 30-m long, 250 μm internal diameter HP-5 column (Hewlett Packard, California, USA). The following conditions were maintained during chromatographic run: carrier gas helium (flow 1.2 ml min^{-1}); oven temperature 200°C held for 2 min, increased to 280°C at the rate of 4°C min^{-1} and held at this temperature for 18 min; injector temperature was 280°C. Temperature of the MS detector was 230°C and that of the transfer line was 280°C. Sterols were identified by their retention times compared with authentic standards and by their mass spectra obtained from a combined GC-MS analysis.

4.6 Statistical analyses

The data were presented as means and standard error of the means. Two-way (I) and three-way analysis of variance (II, III, IV), two-way (I) and three-way (II, III) analysis of covariance and Tukey's multiple range test (I, IV) were used to analyze statistical differences between the treatment means. When the effects of rate of increase in RZT and nutrient supply on PM bond index, amount of free sterols, phospholipid/free sterol ratio and composition of free sterols were tested, three-way analysis of variance was used with temperature treatment, nutrient treatment and duration of treatment as factors (additional data). Logarithmic transformations and angular transformations of square-roots of values were done when necessary. Data were analyzed statistically using SYSTAT 5.0 (1992) for Windows (Systat Inc., Evanston, IL, USA).

5 Results

5.1 Responses of Scots pine seedlings to root zone temperature and nutrient supply

5.1.1 Growth of roots and shoot

In spring, root growth was inhibited by low RZT (I,II). It was almost fully inhibited at RZT of 5°C and was also retarded at RZTs of 13°C or below (Fig. 1 in I, Fig. 2 in II). When air temperature and RZT increased gradually in spring, root growth was highly dependent on development of the RZT sum (Fig. 3B in II). At RZT of 5°C, shoot growth was inhibited by 60% compared with shoot growth at RZT of 12°C and by 62% compared with shoot growth at RZT of 20°C, eventhough the air temperature was constantly 20°C (Fig. 1 in I). Shoot growth was dependent on development of both air temperature and RZT: the shoot sprouting was not initially (first 2 weeks) delayed by slow warming of the root zone, but thereafter the growth of the shoot was retarded in SW-treatment compared to FW-treatment although in these treatments the air temperature sum developed similarly (Fig. 2B in II, Fig. 1). The dependence of root growth on the RZT did not remain the same throughout the growing season (III). Under conditions of high nutrient supply the increment in the root dry mass was high at the end of the growing season, although RZT decreased from 11 to 6°C (Fig. 8 in III). Nor did the RGR of the root dry mass show a clear dependence on RZT during the growing season (Figs. 2A–B). In both nutrient treatments the RGR of the roots

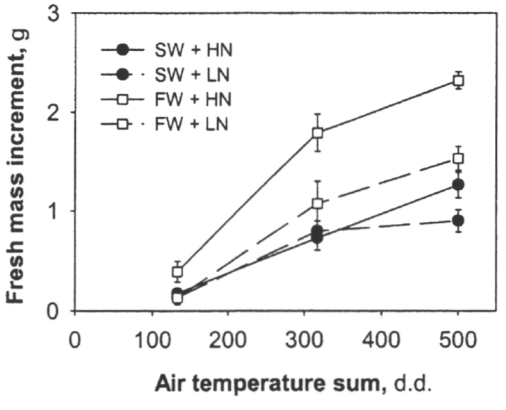

Figure 1. Mean fresh mass increment of the shoot of Scots pine seedlings versus air temperature sum in degree days (d.d.). Seedlings were exposed to slow (SW) or fast (FW) warming of the root zone and grown at high (HN; 3 mM N) or low (LN; 0.5 mM N) nutrient level. Data are means (± SE) of 30 replicates. Redrawn from II.

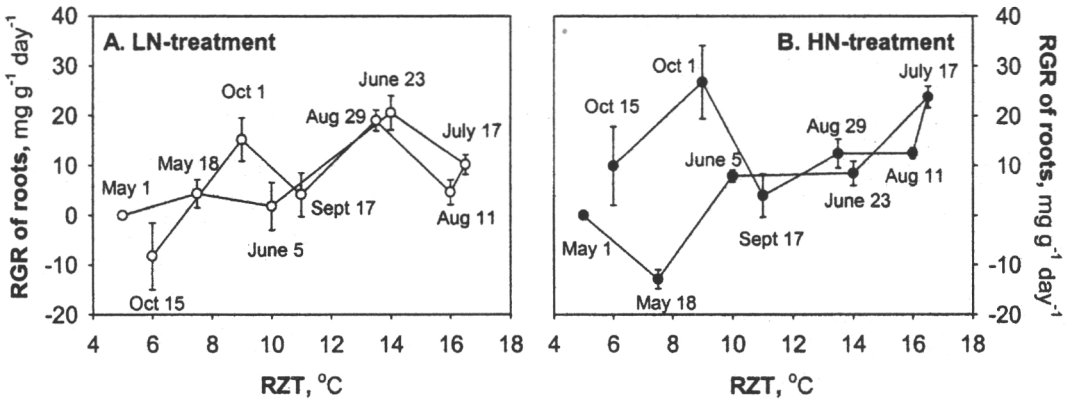

Figure 2. Mean relative growth rate (RGR) of the roots of Scots pine seedlings versus root zone temperature (RZT) during the simulated growing season. The RGR is given as the relative daily increase in root dry mass. Seedlings were grown at low (A. LN-treatment; 0.25 mM N) or high (B. HN-treatment; 2.5 mM N) nutrient level. Data are means (± SE) of 6 replicates, each consisting of 8 sample seedlings. Redrawn from III.

was higher in early October than in early June, although RZT was lower in October (Figs. 2A–B).

The benefit from the high nutrient supply in the growth of the seedlings was apparent only after the RZT had exceeded 13°C in spring (II) or early summer (IV). When this threshold RZT was exceeded, the total fresh mass (Fig. 2 in II, Fig. 2 in IV) and dry mass production (Fig. 3 in III) of the seedlings was promoted by high nutrient supply. Low nutrient supply promoted an increase in the root/shoot ratio (Table 2 in II, Table 1 in IV). The higher seasonal biomass production at high nutrient supply compared to low nutrient supply was due to a longer period of needle elongation, stem diameter growth and root growth (III).

5.1.2 Responses of gas exchange to root zone temperature and nutrient supply in spring

In spring, the RZT had a significant effect on net photosynthesis, transpiration and stomatal conductance of the seedlings (I, II). At RZTs of 12°C and below, net photosynthesis, transpiration and stomatal conductance were lower than at RZT of 20°C (Table 1 in I). At the same time, the internal CO_2 concentration of the needles was high but it decreased during the first ten days of the growing season independently of RZT (Table 1 in I). Net photosynthesis, transpiration and stomatal conductance were increased by the high nutrient supply in spring, and the response in gas exchange became visible earlier and at lower RZT (9°C) than in any other growth parameters measured (Table 3 in II).

5.1.3 Changes in root plasma membranes

Purity of plasma membrane preparations

The PM fraction (upper phase) of the roots, obtained by the aqueous polymer two-phase partition, had a high degree of purity as assessed by the activities of the marker enzymes. Only 1.4–10.3% of the total activity of the marker enzymes of the tonoplast, endoplasmic reticulum and outer mitochondrial membranes, inner mitochondrial membranes and Golgi vesicles was found in the upper phase (Table 2 in I, Table 2). The vanadate inhibition of upper-phase ATPase activity was 88–94%, whereas ATPase activity was unaffected or only slightly affected by inhibitors of non-specific phosphatases, mitochondrial ATPase and tonoplast ATPase (Table 3 in I, Table 3). In addition, ATPase activity of the PM fraction had high substrate specificity for ATP and low specificity for the other substrates tested (Table 4 in I).

Table 2. Distribution of the marker enzyme activities of the [1]tonoplast, [2]inner mitochondrial membrane and [3]Golgi vesicles between the upper and lower phases after aqueous polymer two-phase partition of the microsomal membranes of Scots pine roots. Values represent mean (± SE) percentage of enzyme activity found in each phase in relation to the combined total of both phases. Six randomly sampled samples, each with two replicate assays, were used in the analyses. Data was collected during Experiment 2.

Marker enzyme	Upper phase, %	Lower phase, %
NO_3^--sensitive ATPase [1]	10.3 ± 3.0	89.7 ± 3.0
Cytochrome c oxidase [2]	1.4 ± 0.5	98.6 ± 0.5
Triton X-100 stimulated UDPase [3]	2.4 ± 0.8	97.6 ± 0.8

Table 3. Inhibitor sensitivity of upper phase ATPase activity after aqueous polymer two-phase partition of the microsomal fraction of Scots pine roots. Inhibitors were used for [1]PM-ATPase, [2]non-specific phosphatases, [3]mitochondrial ATPase and [4]tonoplast ATPase. Values are the mean values for specific ATPase activities ± SE of 6 samples, each with two replicate assays, without (control) or with inhibitor. Data was collected during Experiment 2.

Inhibitor	Specific ATPase activity μmol (mg protein $^{-1}$) min^{-1}		% of control
	Control	With inhibitor	
[1]190 μM $NaVO_4$	1.15 ± 0.18	0.12 ± 0.02	12
[2]100 μM $(NH_4)_6Mo_7O_{24}$	1.36 ± 0.17	1.56 ± 0.23	115
[3]1 mM NaN_3	1.01 ± 0.15	1.04 ± 0.12	107
[4]50 mM KNO_3	1.07 ± 0.14	1.04 ± 0.14	100

Real PM-ATPase activity

In spring, the real PM-ATPase activity per unit PM proteins (specific activity) was limited by low RZT: after ten-day exposure the activity was lowest at RZT of 5°C and increased with increasing RZT (Fig. 3 in I). At a constant RZT of 20°C, the real specific PM-ATPase activity decreased during the 10-day period (Fig. 3 in I). When RZT increased slowly in spring (II), the real specific and total activity was low until RZT was 9°C; thereafter the activity increased rapidly, especially in the HN-treatment (Fig. 5 in II, Fig. 3). When the RZT was maintained for 8 days at 18°C, the real PM-ATPase activity decreased also in the HN-treatment (Fig. 5E in II, Fig. 3B).

When studied over the whole growing season (IV), the total activity and the activity per unit fresh mass (FM) were very low until the end of June when an RZT of 14°C was reached (Figs. 3B and 4A–B in IV) and growth of the roots was accelerated (III, IV). At RZTs of 9°C and over the real activity per unit FM of the current roots was much higher than that of previous-year roots (Fig. 3B in IV). The activity of current roots and partly the activity of previous-year roots were favoured by high nutrient supply. The real activity per unit FM was high from mid-July to mid-August when RZT was 16–16.5°C but peaked at the end of September when RZT had already decreased to 9°C (Fig. 3B in IV). The total activity of the root system and current roots reached the highest values at the end of August (LN-treatment) or at the end of September (HN-treatment) at RZTs of 13.5°C and 9°C, respectively (Figs. 4A–B in IV). The PM-ATPase activity of roots was seriously restricted at RZT of 6°C in autumn (Figs. 3B and 4A–B in IV).

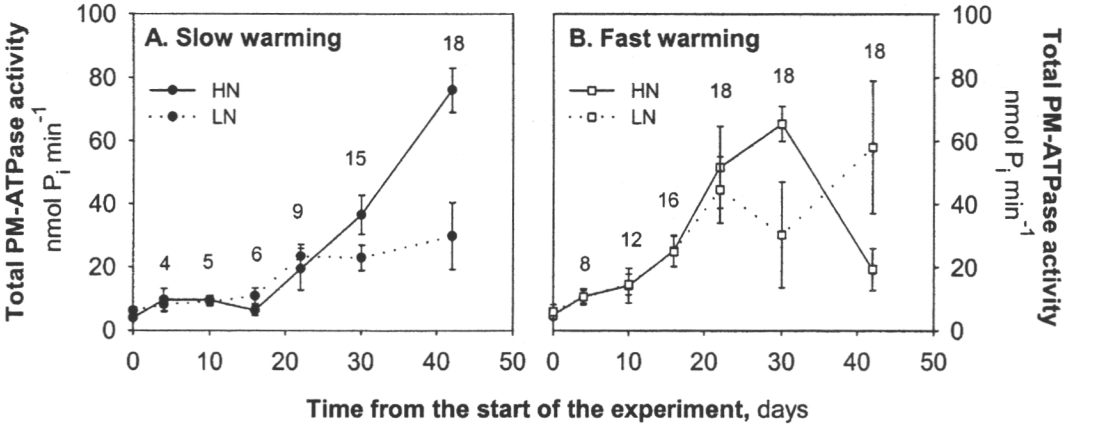

Figure 3. Total PM-ATPase activity of Scots pine roots measured at real RZTs (marked on the figures). Seedlings were exposed to slow (A) or fast (B) warming of the root zone and grown at high (HN; 3 mM N) or low (LN; 0.5 mM N) nutrient level. Data are means (± SE) of 3 replicates. Redrawn from II.

Potential PM-ATPase activity

In spring, the specific potential PM-ATPase activity was not significantly affected by RZT or by nutrient supply (I, II). During the growing season, in the current roots, the PM-ATPase activity per unit FM and total activity were much higher than in the previous-year roots (IV) and with high nutrient supply increased from July to the end of September, while in the previous-year roots the activity was affected by nutrient supply only from the end of August to the end of September (Figs. 3A and 4C–D in IV). At the beginning of October, when RZT decreased from 9 to 6°C, the potential activity of current and previous-year roots per unit FM as well as the potential activity of whole root system decreased rapidly, but remained still higher in the HN-treatment than in the LN-treatment (IV).

Lipid composition of plasma membranes in spring

The amount of PM phospholipids decreased in spring ($p=0.001$) but was not significantly affected by nutrient treatment or by the rate of RZT increment (Fig. 4A–B). The most common fatty acids of PM phospholipids in the roots of Scots pine seedlings were C18:2 and C16:0, which made up 36–46% and 21–32% of the total fatty acids, respectively (I). During the first 10 days in spring, at RZT of 12 and 20°C the proportions of C16:0 and C18:0 increased and those of C18:2 and C18:3 decreased and at RZT of 5°C remained almost unchanged (Table 5 in I). At constant RZT of 5°C the C18:2/C16:0 -ratio and bond index remained almost unchanged, but at constant RZTs of 12 and 20°C declined (Table 5 in I). When RZT increased gradually in spring (Exp. 2, additional data), the bond index decreased during the 42-day period ($p=0.018$), but was not significantly affected by nutrient supply or by the rate of RZT increment (Figs. 5A–B). In the HN-treatment, however, the bond index increased after 16 days exposure to RZTs of 4–6°C and thereafter remained at higher than in the LN-treatment until RZT of 9°C was exceeded (Fig. 5A). The amount of total free sterols did not change in spring (Figs. 4C–D); but due to a decrease in phospholipids, the phospholipid/free sterol ratio decreased (Figs. 4E–F) ($p=0.007$). Over 80% of the total free sterols were sitosterols, and the proportion of stigmasterols and campesterols varied between 5 and 10% (Fig. 6). Of the total free sterols, the proportion of sitosterols decreased ($p=0.001$), and the proportions of campesterol and stigmasterol increased slightly ($p=0.019$ and $p=0.005$, respectively) in spring. These changes were not significantly affected by the rate of RZT increment or nutrient supply, but in the case of campesterols an interaction was found between duration of the treatment and temperature treatment ($p=0.020$). When RZT of 18°C was

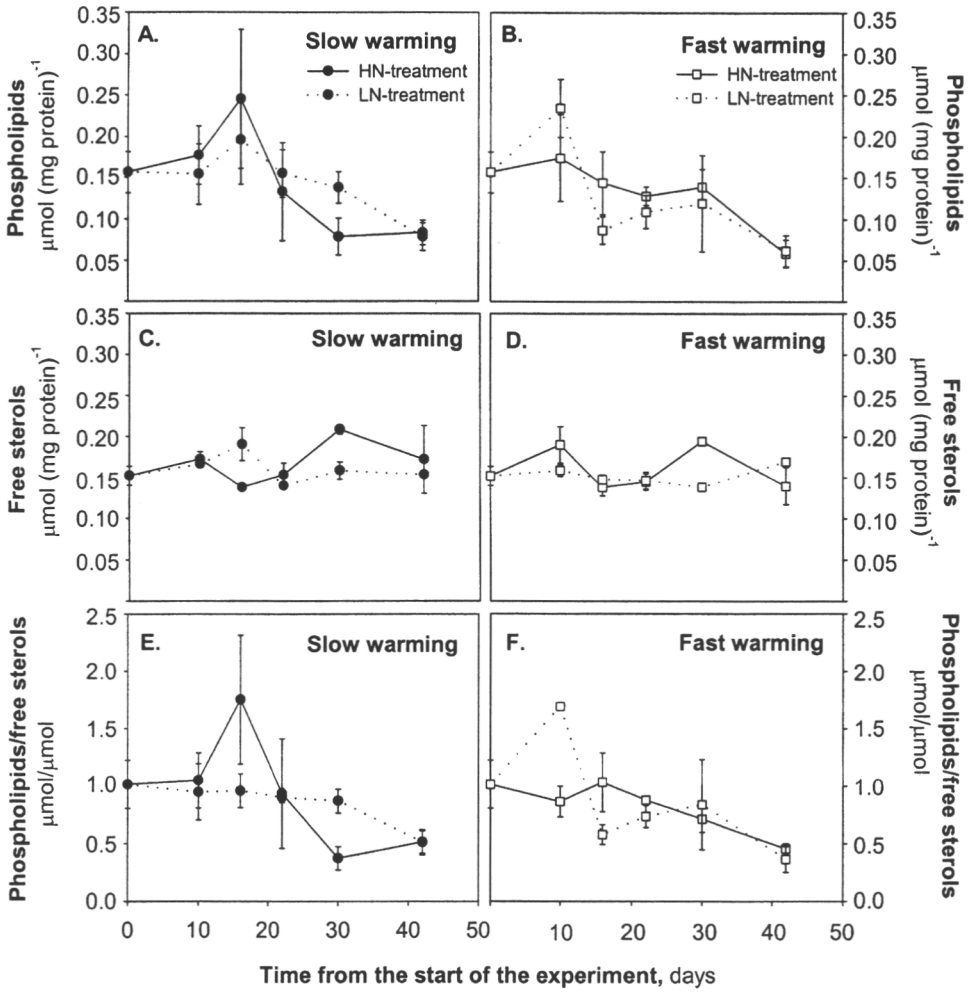

Figure 4. Amount of plasma membrane phospholipids (A–B), free sterols (C–D) and phospholipid/free sterol ratio (E–F) in the roots of Scots pine seedlings. Seedlings were exposed to slow (A, C and E) or fast (B, D and F) warming of the root zone and grown at high (HN; 3 mM N) or low (LN; 0.5 mM N) nutrient level. Data are means (± SE) of 2–3 replicates. Data were collected during Experiment 2 and are presented here as additional data.

maintained for 20 days, the proportion of campesterols decreased to almost the original values at the end of the experiment (Fig. 6D). When RZT increased slowly, the proportion of sitosterols decreased more slowly and that of stigmasterols increased more slowly in the HN-treatment than in the LN-treatment (Figs. 6A and 6E).

5.1.4 *Effect of lipid composition of the plasma membrane on potential PM-ATPase activity*

Even though changes in PM lipid composition were detected, the potential activity of PM-ATPase was not affected by RZT, nutrient supply or duration of treatment (I, II, Figs. 4–6). Nor was any correlation observed between potential PM-ATPase activity and the bond index of phospholipid fatty acids, amount of phospholipids or free sterols, phospholipid/free sterol ratio or free sterol composition (data not shown).

5.1.5 *Nitrogen economy of the seedlings*

In spring the net uptake of nitrogen started at RZT of over 9°C (Fig. 4 in II, Fig. 7 in IV). At lower RZTs, the nitrogen concentration of one-year-old needles and stems decreased (II, IV). Increment of the total nitrogen content of the seedlings was high from the beginning of June to the end of August, especially with high nutrient supply. The decrease in net uptake of nitrogen occurred together with the decrease in growth of the seedlings in September. In the HN-treatment, the increment of nitrogen content still remained high from mid-September to mid-October, when RZT decreased from 11 to 6°C.

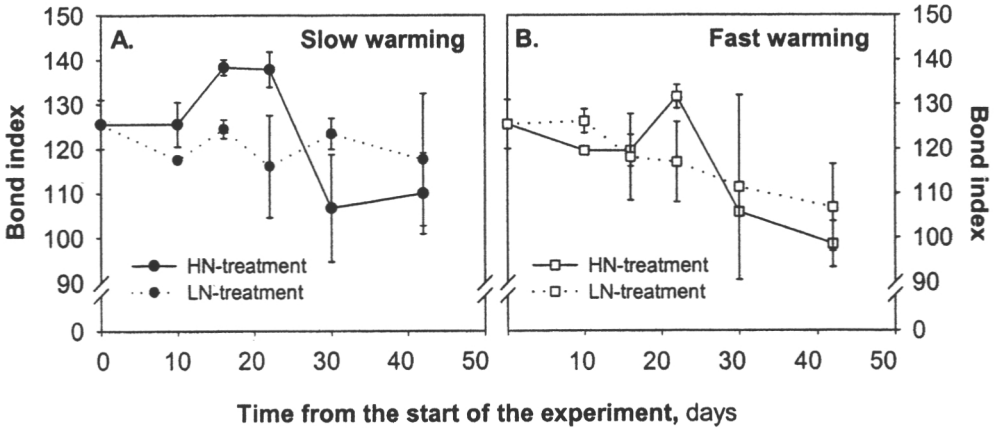

Figure 5. Bond index of plasma membrane phospholipid fatty acids in the roots of Scots pine seedlings. Seedlings were exposed to slow (A) or fast (B) warming of the root zone and grown at high (HN; 3 mM N) or low (LN; 0.5 mM N) nutrient level. Data are means (± SE) of 2–3 replicates. Data were collected during Experiment 2 and are presented here as additional data.

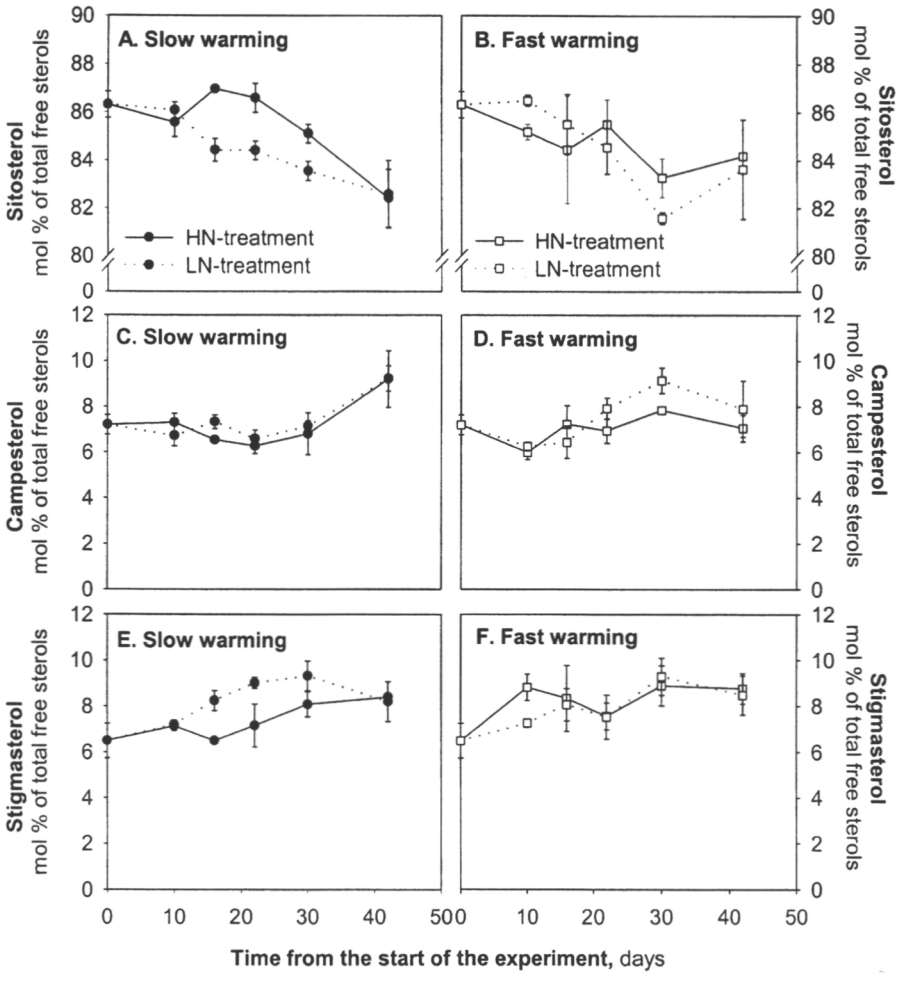

Figure 6. The proportion (mol%) of sitosterols (A–B), campesterols (C–D) and stigmasterols (E–F) of the total free sterols in the root plasma membrane of Scots pine seedlings. Seedlings were exposed to slow (A,C and E) or fast (B, D and F) warming of the root zone and grown at high (HN; 3 mM N) or low (LN; 0.5 mM N) nutrient level. Data are means (± SE) of 2–3 replicates. Data were collected during Experiment 2 and are presented here as additional data.

5.2 Seasonal growth of Scots pine seedlings

The growth of the roots was very slow in May and early June, at the time when the most of the dry mass produced was allocated to the shoot (Figs. 3 and 4 in III). In mid-May, the elongation growth of the shoots started, and it was completed at the end of June regardless of nutrient supply (Fig. 5A in III). Elongation of the current needles started in early June and in the LN-treatment proceeded until the end of August and in the HN-treatment until late autumn (Fig. 5B in III).

In June–July, at the time when shoot growth was intense, about 20–40 % of the dry mass increment was allocated to the root system. The most intense period of root growth was from mid-August to the end of September, at the time when shoot growth was already slowing down. In October, root growth stopped rapidly in the LN-treatment but continued in the HN-treatment (Fig. 2 in III, Fig. 2 in IV).

5.3 Seasonal changes in gas exchange, chlorophyll fluorescence and carbohydrate status as related to nutrient supply

Gas exchange was slow at the beginning of the growing season but increased rapidly when current needles emerged (Figs. 5B and 6A–D in III). In the LN-treatment, the net photosynthesis was about half of that in the HN-treatment in July, at the time when maximum level of net photosynthesis was reached in the LN-treatment. In the HN-treatment, the net photosynthesis remained high until the beginning of October. The internal CO_2 concentration was higher in the LN-treatment than in the HN-treatment from June to the end of the growing season, but at the same time there was no difference in transpiration or stomatal conductance between nutrient treatments (III).

Chlorophyll fluorescence of previous-year needles increased rapidly in early summer, reached maximum at the beginning of August and thereafter decreased. Chlorophyll fluorescence of the current needles also reached the maximum value at the beginning of August and thereafter began to decrease, especially in the LN-treatment (Figs. 6 E and F in III). Thoughout the growing season the chlorophyll fluorescence of the current and previous-year needles was higher in the HN-treatment than in the LN-treatment (III).

In previous-year needles and stem, the starch content increased during May, at the time when growth of the seedlings was low but net photosynthesis was increasing (Fig. 7 in III). In the HN-treatment, the starch content of the previous-year roots, stem and needles decreased rapidly from the beginning of June to mid-July, at the time when root growth was initiated and elongation of the stem and current needles was proceeding. In the LN-treatment, the decrease in starch content was slower or occurred later than in the HN-treatment. In October, the starch content of all organs increased again.

The content of soluble sugars in current needles started to increase in mid-July, at the time when the needles had reached about half of their final length and the elongation growth of the stem was completed (Fig. 7 in III). In the roots, the content of soluble sugars remained constantly low until the end of August and thereafter started

to increase. Non-structural carbohydrates (starch and soluble sugars) did not accumulate in the root system until the end of September (Fig. 3 in III).

5.4 Seasonal changes in the amounts of plasma membrane proteins and PM-ATPase

The amounts of PM proteins and PM-ATPase were higher in current roots than in previous-year roots (Figs. 5 and 6 in IV). The amount of PM-ATPase in the current roots was significantly affected by sampling time but not by HN- or LN-treatment. It was high until mid-September and thereafter decreased (Fig. 6B in IV). In the previous-year roots, the amount of PM-ATPase decreased until mid-July, peaked at the end of August and decreased again to the end of the growing season (Fig. 6A in IV). In September, the amount of PM-ATPase was significantly higher in the HN-treatment than in the LN-treatment.

6 Discussion

6.1 Gas exchange and growth are slowed down by low root zone temperature in spring

Gas exchange

Winter inhibition of photosynthesis, which is characteristic for the boreal conifers (Öquist and Martin 1986), is expressed as a depression of the photochemical effeciency of PSII (Strand and Lundmark 1995). Trees recover from winter inhibition of photosynthesis in spring and early summer (Troeng and Linder 1982, Strand and Lundmark 1995, I, II, III). In the present experiment, net photosynthesis of the seedlings was limited by non-stomatal factors probably due to winter inhibition, not by low RZT, immediately after winter storage (I). During recovery, net photosynthesis increased and gradually became dependent on RZT (I,II). Net photosynthesis, transpiration and stomatal conductance were low at RZT of 5°C, and still at temperatures of 9–12°C (I, II). At that time, the stomatal limitation of net photosynthesis was evident and was seen as a lowered rate of transpiration and stomatal conductance and low internal CO_2 concentration at low RZT (I).

The limitation of net photosynthesis by low RZT has been demonstrated earlier in Scots pine (Lippu and Puttonen 1991, Vapaavuori et al. 1992). The lowered rate of gas exchange at low RZTs is probably due to several factors including limited rate of water and nutrient (I, II, IV) uptake, limitation of root growth (I, II, IV) as well as low demand for photosynthates (III). At RZTs below 12°C, the water potential of Scots pine shoots and roots is low (Vapaavuori et al. 1992), which might be due to the high viscocity of water and the high resistance of roots to water transport (Kaufmann 1977). The high resistance of roots to water transport at low RZTs is caused by delayed formation of new unsuberized roots (Häussling et al. 1988, Wan et al. 1999, I, II), low permeability of the PM lipid bilayer (Finkelstein 1987) and possibly also by limited activity of the PM water channels, aquaporins (Wan and Zwiazek 1999). At low RZTs the lowered rate of photosynthesis may also be due to reduced demand for photosynthates for root growth. Because at moderate stress photosynthesis is reduced less than growth of the seedling (Wardlaw 1990), photosynthates accumulate in needles and stem because pro-

duction is greater than demand (Kursanov 1984, III). Accumulation of an excess of photosynthates in the form of starch in chloroplasts can also reduce photosynthesis (DeLucia 1986).

Growth of the seedlings

The inhibition of initial root growth by low RZT has been demonstrated earlier in several pine species (Bowen 1970, Larson 1967, Grossnickle and Blake 1985, Andersen et al. 1986, Lopushinsky and Max 1990) including Scots pine (Prokushkin 1982, Vapaavuori et al. 1992, Ryyppö et al. 1994, Lyr and Garbe 1995). This was also seen in the present investigation in which root growth was clearly dependent on RZT in spring, being strongly restricted at RZT of 5°C and limited at RZTs below 13°C (I, II). For one-year-old Scots pine seedlings, the threshold temperature for the appearance of new root tips and root elongation in spring has been shown to be 5°C and 8–12°C, respectively (Vapaavuori et al. 1992); and the results of the present study confirm these observations. It has been suggested that the lower threshold temperature for root-tip initiation compared to that for root elongation is due to separate factors controlling these processes. Root-tip initiation can be energized by stored carbohydrates; but for elongation growth of the roots current photosynthates are necessary (van den Driessche 1978, Andersen et al. 1986, Vapaavuori et al. 1992). In addition to reduced rate of net photosynthesis with low RZT (I, II), in Scots pine seedlings the translocation of current photosynthates to roots is also reduced at RZTs of 5–8°C (Lippu 1999). The limited rate of translocation of photosynthates to roots at low RZTs can be partly due to low PM-ATPase activity (I, II), which is needed for phloem unloading of photoassimilates in roots. Furthermore, PM-ATPase is also needed for the extension growth of the root cell, which at low RZTs is limited (Pritchard et al. 1990). Reduced extension growth leads to low growth demand of the roots for carbohydrates, which, in turn, at low RZTs can limit translocation of photosynthates. In addition to reduced rate of cell extension growth, the inhibition of root growth can be due to prolonged cell-division cycle (Barlow 1987). In spring, the rate of root biomass production is related to the cumulative temperature sum of the root zone (II). Because in boreal forests the RZTsum normally develops more slowly than the air temperature sum, a lag in root growth, compared to shoot growth, seems to be characteristic for Scots pine seedlings planted in springtime.

Shoot growth was not as sensitive to RZT as root growth; however, at constant RZT of 5°C it was limited (I) and when the RZT increased slowly in spring it was delayed (II, Fig. 1). Thus, shoot growth in spring is determined both by air temperature and RZT in spring.

The decreased shoot growth was probably due to delayed formation of current needles (unpublished data, Lyr and Garbe 1995), because the timing for bud burst and duration of shoot sprouting seem to be strongly influenced by air temperature (Vapaavuori et al. 1992, Lyr and Garbe 1995).

The benefit from high nutrient supply was seen as accelerated growth of the roots and shoot only after the RZT was increased to 13°C (II). This is in agreement with an earlier field study with *Pinus strobus* which showed that application of fertilizers has no effect on growth of seedlings in cold soil (Brand and Janas 1988).

6.2 Changes in lipid structure of the root plasma membrane are necessary for an active root system

In this study, no significant changes in PM lipid composition occurred at RZT of 4–5°C (I, Figs. 4A, 4C, 4E, 5A), which indicates that RZTs of 4–5°C may be too low for the cold deacclimation process of the root PM. When the RZT increased slowly in spring from 5 to 9°C, the bond index increased in the HN- but not in the LN-treatment (Fig. 5A). The higher bond index of PM phospholipids in the HN-treatment indicated higher fluidity of PM in the HN-treatment compared to the LN-treatment (Thompson 1984).

At the same time, changes also occurred in the relative amounts of free sterols. The sitosterol/stigmasterol ratio of free sterols was higher in the HN-treatment than in the LN-treatment (Fig. 6A and 6E). This is consistent with data from nitrogen-deprived wheat roots. Carvajal et al. (1996a) found a higher sitosterol/stigmasterol ratio in the PM of control roots than in nitrogen-deprived roots, and a decrease of PM fluidity was observed in the latter roots. However, the roles of different sterol classes in regulating PM properties are still obscure. It has been suggested that sitosterols are able to order the fatty acid chains of the phospholipid bilayer (i.e. are able to decrease fluidity), while stigmasterols have been found to exhibit less ordering ability (Grandmougin-Ferjani et al. 1997). This suggestion does not agree with the idea that the decrease in PM fluidity is linked to the decrease in the sitosterol/stigmasterol ratio in nitrogen-deprived roots. Since the changes in PM lipid composition may have complex effects on the membrane fluidity and no fluidity measurements were made in this study, differences in the PM fluidity between nutrient treatments cannot be evaluated. In addition, because the bond index also fluctuated at higher RZTs (Fig. 5B), these results in the context of low temperature acclimation must be interpreted with caution. In spite of these facts, it is worth noting that an increase in phospho-

lipid fatty acid unsaturation and an increase in sitosterol/stigmasterol ratio after long-term exposure to low RZTs might be the coping mechanism that limits the negative effects of low RZT under conditions of high nutrient supply.

Differences in PM lipid structure between the LN- and HN-treatments may explain some of the changes found in gas exchange of the seedlings at RZT of 9°C (II). In this study, the benefit of net photosynthesis with a high nutrient supply became evident when RZT of 9°C was exceeded (II). At that time, net photosynthesis was 1.5 times higher in the HN-treatment than in the LN-treatment, but the nitrogen concentration of needles was only slightly higher in the HN-treatment. Therefore, higher transpiration and stomatal conductance (II) may indicate that under conditions of high nutrient supply more efficient water uptake enables higher net photosynthesis. According to earlier studies, high nutrient supply can increase hydraulic conductance of the root system as well as the PM fluidity of the roots (Radin 1990, Carvajal et al. 1996b). A high supply of nutrients can promote water uptake by increasing the activity of aquaporins in root PMs (Carvajal et al. 1996a), probably due to structural interaction between bilayer lipids and aquaporins (Carvajal et al. 1996b), while an increase in unsaturation of PM fatty acids seems to increase the water permeability of the bulk lipid bilayer (Markhart et al. 1979, 1980).

A decrease in the phospholipid/sterol ratio and an increase in fatty acid saturation have been reported to contribute to a reduction of bilayer fluidity (Thompson 1984). A decrease in the phospholipid content, an increase in phospholipid fatty acid saturation and a decrease in the phospholipid/free sterol ratio (Table 5 in I, Figs. 4 and 5) occurred at RZTs that allowed root growth (I,II). These changes are consistent with earlier observations in aboveground organs of extremely cold-hardy trees (Yoshida 1986, Sutinen 1992). At RZTs of 12 and 20°C, the proportion of C18:3 of phospholipid fatty acids decreased in the root PMs of Scots pine seedlings. In contrast to these results, during cold deacclimation an increment in the proportion of C18:3 takes place in the needles of Austrian pine (*Pinus nigra*) and red pine (*Pinus resinosa*) (Sutinen 1992) and in living bark tissue of Mulberry tree (*Morus bombysis*) (Yoshida 1986). Fatty acid composition of PM phospholipids in soybean roots have been found to differ depending on the developmental stage of the root tissue; in particular, the proportion of C18:3 is higher in mature root tissue than in meristematic root tissue (Whitman and Travis 1985). When root growth is accelerated in spring, the proportion of meristematic tissue in the root system increases. Therefore, in addition to the saturation of the phospholipid fatty acids that is linked to cold deacclimation, the decrease of C18:3 in root PMs of Scots pine seedlings might be associated with changes in the fatty acid composition of phospholipids that are linked to development.

The decrease in sitosterols and the corresponding increase in campe– and stigmasterols are changes opposite to those occurring during cold acclimation (Uemura and Yoshida 1984, Lynch and Steponkus 1987, Palta et al. 1993), which indicates that these changes are related to metabolic adjustment of the PM to increasing RZT. However, because current roots were not separated from older roots in this study, it is not possible to differentiate clearly the lipid changes related to the developmental stage of the roots from changes that may result from metabolic adjustment of PM to increasing RZT. It is obvious that some changes observed in free sterols after long-term exposure to RZT of 18°C (FW-treatment) might be linked to developmental changes instead of metabolic adjustment of the PM to increasing RZT. Travis and Berkowitz (1980) reported that the campesterol level in the root PM decreases with the differentiation of root tissue, which can explain the similar levels of campesterols in the fast warming treatment at the beginning and end of the experiment (Fig. 6D).

6.3 Hydrolytic activity of root PM-ATPase is not modulated by bulk lipid structure

Lipid modulation has been proposed as one of the mechanisms by which the activity of PM-ATPase can be regulated (Carruthers and Melchior 1986, Cooke and Burden 1990). The results of the present study, however, indicate that there is no clear relationship between potential PM-ATPase activity and bulk lipid composition (I, Figs. 5C and 5F in II, Figs. 4, 5 and 6), suggesting that the PM-ATPase activity of Scots pine roots is not regulated by the bulk membrane composition. Similarly, in the roots of herbaceous species, the PM-ATPase activity appeared to be independent of the bulk PM sterol/phospholipid -ratio (White et al. 1990) and of the phospholipid fatty acid saturation (Burgos and Donaire 1996). Due to the lateral heterogeneity of the PM, changes in the bulk lipid composition do not necessarily reflect events in the lipid composition surrounding the enzyme (Cooke and Burden 1990). Therefore, comparison of the PM-ATPase activity and bulk composition of the membrane lipids, as done in this study, does not take into account the individual lipid/protein interaction, which may affect the activity of the enzyme. Furthermore, the lipid composition of the PM can affect coupling factor of the PM-ATPase, which was not examined in this study. In simultaneous measurements of the H^+-pumping and hydrolytic activity of PM-ATPase of corn roots, Grandmougin-Ferjani et al. (1997) found that H^+-pumping is more sensitive to the sterol environment than ATP hydrolysis is.

6.4 Seasonal growth of shoot and roots in Scots pine seedlings is episodic

The results of this study show that the seasonal growth of the stem, needles and roots of one-year-old Scots pine seedlings occurs in phases, which leads to episodic growth of the shoot and roots (III). Episodic growth of shoot and roots has also been found in the seedlings of other northern pine species (Cannel and Willet 1976, Drew 1982). Drought, low soil temperature and lack of currently formed photosynthates for root growth have commonly been proposed as factors that suppress root growth during the growing season (Ritchie and Dunlap 1980, Deans and Ford 1986, Dougherty et al. 1994, Eissenstat and van Rees 1994). It has been suggested that the mid-summer depression of root growth is caused by drought (Sword et al. 1997). Since root growth had an episodic growth pattern in hydroponic culture where the drought-effect was excluded, apparently soil moisture is not the principal factor limiting root growth of Scots pine seedlings during the growing season.

Root growth of the seedlings was very slow in May and early June, when most of the structural dry mass (= total dry mass – content of soluble sugars and starch) production was allocated to the elongating stem (III). At this time, the production of photosynthates exceeded the demand, and photosynthates accumulated in the previous-year needles. This indicates that the low rate of root growth in spring is not due to limited rate of production of current photosynthates. Rather, it is due to limited rate of translocation of current photosynthates to the roots (Andersen et al. 1986, Lippu 1999), which may be caused by low metabolic activity, and thus low sink strength of the root system (Hurewitz and Janes 1983, IV). At the end of the June, at which time RZT was increased to 14°C, growth of the current roots was accelerated. The formation of new roots leads to increasing PM-ATPase activity of the whole root system, because the PM-ATPase activity of the current roots is much higher than the activity of the previous-year roots (IV). The higher PM-ATPase activity in current roots compared to the previous-year roots is due to the greater amount of the enzyme (IV). Furthermore, a greater amount of PM proteins in current roots compared to the previous-year roots (IV) is an indication of the higher biological activity of the current roots (Morris and Clarke 1987).

The increased rate of root growth and increased PM-ATPase activity of the root system facilitated water uptake (Häussling et al. 1988) and nitrogen net uptake (IV), which led to an increase in gas exchange (III). Elongation of the current needles started at almost the same time as the growth of the current roots (III). In the early stages of development, net photosynthesis of the current needles is low and they con-

tain high sink activity of current photosynthates (Troeng and Linder 1982, Lippu 1999). It has been estimated that the carbohydrate production of the current needles is high enough to meet the demand of other organs only when the half of the final length of the needles has been reached (Ericsson 1978). If the current photosynthates are necessary for root elongation (Vapaavuori et al. 1992), then the root growth of Scots pine seedlings is dependent on photosynthates produced by the previous-year needles until the end of July when current needles have reached half of their final length (III). During the time of intensive needle elongation, only 15–36% of the total dry mass increment was allocated to roots, with no net accumulation of soluble sugars and starch to the roots (III). Structural growth of the roots, as well as net accumulation of soluble sugars and starch, was most intense after the period of intensive needle elongation, at the time when most of the shoot growth had taken place (III). This confirms the suggestion that root growth is slowed down during the time of intensive shoot growth because the growing shoot is a strong consumer of photoassimilates (Lyr and Hoffmann 1967, Mattson 1986).

6.5 Nutrient supply affects growth but not the growth rhythm of Scots pine seedlings

The nutrient supply does not significantly affect the seasonal pattern of dry mass allocation to the roots (III). However, the total growth of the root dry (III) and fresh (IV) mass is favoured by a high supply of nutrients, mainly due to the later cessation of root growth under conditions of a high supply of nutrients. The delayed cessation of fine root growth by fertilization has been observed earlier in Scots pine (Persson 1980) and loblolly pine (*Pinus taeda*) stands (Sword et al 1996). High nutrient supply stimulates the PM-ATPase activity of the roots (Kuiper et al. 1991, II,IV) leading to efficient nitrogen uptake of the seedlings (IV). Nitrogen enrichment in the needles (III) increased the net photosynthetic rate of the seedlings (Linder and Rook 1984, Paquin et al. 2000, II,III), resulting in more carbon allocation for needle production (Field and Mooney 1986, Rikala and Huurinainen 1990, Lambers et al. 1998, III). High nitrogen concentration of the needles increased the rate of net photosynthesis by increasing the efficiency of the PSII (III) and possibly the amount of RuBP carboxylase (Gezelius 1986). Furthermore, the low efficiency of net photosynthesis under conditions of low nutrient supply can be the result of a low concentration of chlorophyll in the needles (Gezelius 1986).

It is well documented that plants allocate relatively more biomass to roots and less to the shoot in response to nutrient shortage, partic-

ularly nitrogen and phosphorus (Wilson 1988, Levin et al. 1989, Lambers et al. 1998), and this was also seen in the present study (II,IV). Van der Werf and Nagel (1996) presented a hypothetical model to account for the positive effect of nitrogen shortage on biomass allocation to roots. According to this model, an early response of a plant to nitrogen shortage is a decrease in the synthesis and export of cytokinins (Salama and Wareing 1979, Kuiper 1988, Kuiper et al. 1989). This leads to the decreased rate of needle growth (Lambers et al. 1998, III) and hence to the accumulation of carbohydrates in the needles (III), which, in turn, leads to down-regulation of photosynthesis (III). The increased level of carbohydrates in the needles, however, may imply that a larger fraction of photosynthates is available for translocation to the roots. In the roots, a high level of sugar may induce genes encoding for respiratory enzymes and possibly other enzymes (Farrar 1996), resulting in enhanced root growth in relation to shoot growth.

6.6 Temperature dependence of root growth and PM-ATPase activity varies during the growing season

It is generally known that root growth and other enzymatically driven processes in the roots are limited by low RZT. To ascertain the minimum, maximum or optimal RZTs for root growth and activity, the temperature dependence of root growth and metabolic activity has generally been observed in relatively short studies at a certain stage of development. However, it has recently been discussed (Fitter et al. 1998, Pregitzer et al. 2000) that there is sometimes little correlation between temperature and root growth, which is probably related to the phenological pattern of root growth and to limitations of root growth by other growth-limiting factors. In the present study, it was observed that the response of root growth to RZT does not remain the same throughout the growing season; and consequently, RZTs that inhibit or retard root growth in spring may be favourable for root growth in autumn (III, Fig. 2A–B). This may also indicate that low RZT may not be the primary factor limiting root growth in spring. Furthermore, the sensitivity of PM-ATPase activity to RZT seems to be dependent on the stage of seedling development (IV). At RZTs over 9°C, neither root PM-ATPase activity per unit fresh mass nor total PM-ATPase activity can clearly be shown to be dependent on temperature (Fig. 5 in II, IV, Fig. 3). It can be suggested that at RZTs which favour root growth and root PM-ATPase activity these activities are more dependent on the growth demand for nutrients than on RZT *per se*.

7 Conclusions

This research was conducted in order to obtain better knowledge of the seasonal pattern of root growth and activity and information on whole-tree physiological responses of one-year-old Scots pine seedlings to RZT and nutrient supply. Better knowledge might help reforestation practises to find out the periods when the root growth and activity is high enough to lead into rapid establisment of seedlings to the planting site. This study was done in hydroponic culture, which allows plants free access to water and available nutrients and thus may permit more optimal growing conditions compared to solid growth medium. Furthermore, hydroponic cultures do not favour the growth of mycorrhizas that are normally involved in water and nutrient uptake from forest soil. These facts should be taken into account when the results of this study are compared to studies where mycorrhizal Scots pine seedlings grown in solid growth medium are used. In spite of the artificial growing conditions used, the following conclusions from the study could be helpful for reforestation practise.

In spring, low RZT limited gas exchange, root PM-ATPase activity and nitrogen net uptake and delayed the cold deacclimation processes of root PM lipids in one-year-old Scots pine seedlings. This led to inhibition of root growth at RZT of 5°C and to delay of root growth at RZTs below 13°C. Shoot growth was not as sensitive to RZT as root growth, but was to some extent delayed by low RZT. Scots pine seedlings were able to recover from the negative effects of low RZT as the RZT increased and the recovery could be speeded up by supporting the high fertility of root zone. Already at RZT of 9°C, greater gas exchange was seen in the HN-treatment compared to the LN-treatment, although the benefit from high nutrient supply was not seen in the growth of the seedlings until RZT of 13°C was reached. At RZT of 9°C, the higher net photosynthesis in HN-treatment compared to LN-treatment may be due to more efficient water uptake facilitated by changes in lipid composition of the PM. In field conditions, where water availability is not as high as in hydroponic culture, delayed formation of new roots may have a prolonged negative effect on establishment of the seedlings to the planting site.

The present study provides evidence that root growth of one-year-old Scots pine seedlings is strongly related to the shoot phenology. Growth of the roots was slow in spring and early summer when most of the structural biomass was allocated to the stem. At that time, photo-

synthates accumulated in the shoot, which indicates that the low rate of root growth was not due to limited production of current photosynthates. Rather, slow root growth was caused by low metabolic activity of the root system in spring. Root growth was accelerated at the end of June, when RZT of 14°C was reached. Rapid formation of the current roots led to increased PM-ATPase activity of the root system and net uptake of nitrogen, because the current roots have a higher metabolic activity than previous-year roots. In mid-summer, during the time of intense needle elongation, root growth was suppressed, presumably due to the competition between growing shoot and roots for photosynthates. The most intense period of root growth was at the end of the growing season after the period of intense needle elongation. At the end of the growing season, growth of the roots and the PM-ATPase activity of root system remained high until the RZT fell below 9°C. Nutrient supply did not affect the seasonal pattern of root growth, but the total growth of the roots, root/shoot ratio and activity of the roots were affected by nutrient supply.

According to the results of this study, the dependence of root growth and PM-ATPase activity on RZT do not remain the same during the growing season. RZTs that limit root growth and PM-ATPase activity in spring can be favourable for root growth and activity at the end of the growing season. During the growing season, growth and PM-ATPase activity of the roots are controlled by several factors, of which RZT is not always the primary one.

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Responses of Scots pine seedlings to low root zone temperature in spring

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The effects of root zone temperature (RZT) on growth, gas exchange, H^+-ATPase (EC 3.6.1.3; PM-ATPase) activity and fatty acid composition of plasma membrane (PM) phospholipids in the roots of one-year-old seedlings of Scots pine (*Pinus sylvestris* L.) were studied for 10 days during flushing in spring. Nursery-grown seedlings were transferred to cold storage (−5°C) in mid-October, thawn in a cold room at 5°C in May, and transferred to hydroponic cultures at an air and root zone temperature of 5°C for a 3-day adjustment period. The experiment started when the RZT was changed to either 5, 12 or 20°C and the air temperature was increased to 20/15°C (day/night). RZTs of 5 and 12°C were suboptimal for root growth, and also shoot growth was suppressed at 5°C. The degree and rate of phospholipid fatty acid saturation in the PM of roots was highest at RZT of 20°C and intermediate at 12°C, while no change in the degree of saturation occurred at 5°C. PM-ATPase activities, measured at 5 and 12°C (real activities) were severely temperature-limited, but the increasing potential activities (measured at 38°C) at these RZTs indicated delayed deacclimation of the root system. At RZT 20°C, the decline of C18:2/C16:0 ratio in combination with decreasing potential and real PM-ATPase activities indicated, instead, fast deacclimation of the root system. Net photosynthesis of the seedlings was limited by non-stomatal factors at the beginning of the experiment, but recovered from winter inhibition and later became limited by low stomatal conductance at RZTs of 5 and 12°C. Instead, at 20°C the net photosynthesis increased with increasing stomatal conductance during the experiment. We conclude that low RZT suppresses growth of roots and such changes in the PM as are needed for efficient uptake of water and nutrients. This, in turn, limits net photosynthesis and, thus, the availability of photosynthates for root growth in spring. On the other hand, a rise in RZT can rapidly induce the structural and functional changes in PM of the roots that are needed for the efficient gas exchange and growth of the Scots pine seedlings.

Key words – Fatty acids, gas exchange, H^+-ATPase, phospholipids, *Pinus sylvestris*, plasma membrane, roots, Scots pine, temperature.

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Introduction

In Finland conifer seedlings are typically outplanted prior to bud break in May, when soil temperature is below 10°C (Finnish Meteorological Institute 1979) and suboptimal for root growth (Andersen et al. 1986, Nambiar et al. 1979, Ritchie and Dunlap 1980). Rapid formation of permeable, unsuberized roots is important for successful establishment of newly planted seedlings, because new roots provide the most efficient uptake of water and nutrients (Häussling et al. 1988). Resistance

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to the flow of water into the roots is enhanced in cold soil, not only because of the suberized state of the old roots and the high viscosity of water, but also due to the composition of root cell membranes (Chung and Kramer 1975, Grossnickle 1988, Kaufmann 1975, 1977). The cell membranes of overwintering plants undergo seasonal changes connected with cold acclimation and deacclimation (Uemura and Steponkus 1994, Uemura and Yoshida 1984, Yoshida 1986). Changes in lipid unsaturation have an important role in determining the thermotropic properties of the plasma membrane (PM) and, thus, the competence of the cells for maintaining metabolic activity, especially in plant species capable of extreme cold hardiness of cells (Yoshida 1984). A shift of the PM towards a phospholipid-enriched state during cold acclimation, and a decline in phospholipids during deacclimation, may be observed as general features of cold hardiness in these plants (Uemura and Yoshida 1984).

Modification of the chain length and changed desaturation of the phospholipid fatty acids in the PM have been observed in connection with the changes in the amount of phospholipids (Sutinen 1992, Uemura and Yoshida 1984, Yoshida 1984). The modifications are manifested as increases in the proportion of di-unsaturated species of phosphatidylcholine and phosphatidylethanolamine (Uemura and Steponkus 1994). Furthermore, seasonal changes in the degree of fatty acid saturation may modulate the activity of PM bound H^+-ATPase (PM-ATPase) (Palta and Weiss 1993), which serves as the major ion pump in plants and energizes PM for secondary active uptake and transport of ions and nutrients. PM-ATPase has been described also as a key regulatory enzyme in cellular events during hardening and dehardening (Sussman and Surowy 1987, Sutinen 1992). Rapid growth of roots and differentiation of root cells facilitate the uptake and transport of ions by increasing the amount of PM-ATPase in the root tissues enriched in the enzyme (Roldán et al. 1991). Large amounts of PM-ATPase have been found in certain root cells, e.g. in epidermis, endodermis and central cylinder, which have a vital role in ion uptake and transport (Parets-Soler et al. 1990, Villalba et al. 1991).

The threshold temperature for root tip initiation is 5°C and that for further lengthening of the tips in Scots pine seedlings is between 8 and 12°C in spring (Vapaavuori et al. 1992). At such low RZTs, phospholipid fatty acids extracted from a microsomal membrane fraction of Scots pine roots retain a high degree of unsaturation (Ryyppö et al. 1994). On the other hand, at an RZT that promotes root growth, changes in the degree of fatty acid saturation and in metabolic activity in the roots can be rapid. Furthermore, high metabolic activity in the roots, measured as potential PM-ATPase activity of the microsomal fraction depends, in the long term, on the growth of new, unsuberinized roots (Ryyppö et al. 1994).

The most obvious changes in the H^+-ATPase activity in the microsomal fraction of roots occur during the first week of flushing (Ryyppö et al. 1994). In the present study we provide more detailed information on the early effects of low RZT and high air temperature on deacclimation and root growth. The effects of RZT on root deacclimation were assessed by determining the degree of fatty acid saturation in the plasma membrane phospholipids. At the same time, the effects of RZT on growth initiation and metabolic activity in the roots were measured as the activity of PM-ATPase. Finally, to study the effects of low RZT in combination with high air temperature on the recovery of photosynthesis from winter inhibition, gas exchange of the seedlings was measured. Hydroponic cultures were used for accurate control of RZT; allowing at the same time free access of water and nutrients and facilitating the sampling of the roots.

Abbreviations – ATP, adenosine triphosphate; BHT, butylated hydroxytoluene; PM, plasma membrane; PM-ATPase, plasma membrane H^+-ATPase; PMSF, phenylmethylsulfonyl fluoride; RZT, root zone temperature.

Materials and methods

Plant material and experimental treatments

One-year-old seedlings of Scots pine (*Pinus sylvestris* L.), sown 1992-05-14, were grown at a density of 620 seedlings m^{-2} in fertilized peat (Finnpeat M6, Kekkilä Corp., Tuusula, Finland) at the Suonenjoki Research Station (62°05′N, 27°00′E, 130 m above sea level), using normal nursery practices. The seeds were from a seed orchard (Toivakka 62°05′N, 26°10′E) established with material from different sites located in Finland between 61°70′N and 64°20′N. During the growing season, the seedlings obtained 6.9 g N m^{-2}, 4.4 g P m^{-2} and 1.5 g K m^{-2} (plus micronutrients) in the irrigation water (1992-06-16–1992-09-07). In mid-October the seedlings were transferred to cold storage (−5°C) for 5 months, and for the second growing season they were thawed in darkness in a cold room at 5°C for 7 days. After thawing, the rootballs were washed free from peat with cold tap water. After washing, 1 128 seedlings were divided among twelve 40-l containers (94 seedlings per container). The vessels were filled with aerated tap water and moved to a growth chamber (Weiss, type 10 Sp/5 DU-Pi, Lindenstruth, Germany). The seedlings were kept at 5°C and in continuous light (PAR 125 μmol m^{-2} s^{-1}, HQI-T250W/D 3994/003 lamps, Osram, Berlin, Germany) for the first three days. The 10-day experiment started when the tap water was replaced by nutrient solution (Ingestad and Lund 1986), which was thermostated to 5, 12 or 20°C (4 containers per temper-

ature) by circulating through cryostats (MGW, Lauda, Germany). The daily photoperiod during the experiment was 18 h (PAR 300 μmol m^{-2} s^{-1}, HQI-T250W/D 3994/003 lamps, Osram), air temperature 20/15°C (day/night) and RH 40–45%/70–75% (day/night).

Growth determination

Growth of the seedlings was determined by the displacement method (Burdett 1979), by weighing 10 seedlings from each container at the beginning and end of the 10-day experiment. The mean initial volume of the roots was $1\,043 \pm 25$ mm^3 ($\pm$SE, n = 120) and the mean weight of the shoots $2\,115 \pm 51$ mg ($\pm$SE, n = 120).

Gas exchange measurements

Gas exchange of the whole shoot was measured with a closed system infrared gas analyzer (Li-Cor 6200, Li-Cor, Lincoln, NE, USA) on days 1, 4 and 10. During the measurements, the RZT was maintained at 5, 12 or 20°C and shoot temperature at 22°C. Irradiance was 550 μmol m^{-2} s^{-1} PAR (cool beam halogen lamps FTY GX5.3, Sylvania, Erlangen, Germany) and RH 40–50%. Gas flow in the closed system was adjusted through the desiccant, so that RH in the cuvette was constant and CO_2 concentration decreased at a constant rate. After constant RH and a CO_2 concentration of about 370 μl l^{-1} were obtained, gas exchange was measured for a period of 1 min. Gas exchange rates were calculated on the basis of the silhouette areas of the shoots, which were measured by a video camera connected to a graphic monitor (Vapaavuori et al. 1992).

Isolation of plasma membranes

Material for PM isolation was collected on days 0, 1, 4, 6, 8 and 10. Each sample consisted of the roots of 12 seedlings from one container; thus four replicate samples per RZT were collected on each sampling day. The samples were frozen in liquid nitrogen and stored at −80°C.

Microsomes were prepared as described by Ryyppö et al. (1994) and the PM-enriched fraction was obtained by aqueous polymer two-phase partitioning (Iswari and Palta 1989, Sutinen 1992). The final concentrations of both Dextran T-500 (M_r 500 000) and polyethylene glycol (M_r 3 500) in the phase systems (in 0.25 *M* sucrose-phosphate buffer, pH 7.4) were 5.7% and that of NaCl 0.3 *M*. The upper PM-phase was separated from the lower phase containing endomembranes by centrifugation in a swing-out rotor at 1 500 *g* for 15 min at 4°C. Both membrane fractions were washed once and resuspended in phosphate-free buffer (0.53 *M* sucrose, 5 m*M* MOPS-NaOH, 1 m*M* EGTA, 10 m*M* KCl, 0.2 m*M* PMSF, 10 mg l^{-1} BHT and 1 m*M* DTT, pH 7.3), then centrifuged in a fixed-angle rotor at 48 000 *g* for 1.5 h at 4°C. The pellets obtained by centrifugation were resuspended in the phosphate-free buffer and stored at −80°C.

PM-ATPase activity and assays of marker enzymes

PM-ATPase (EC 3.6.1.3) activity was measured as described by Ryyppö et al. (1994), as the amount of P_i released from ATP hydrolysis (Hodges and Leonard 1974) in the presence of 5 m*M* NaN_3, 100 m*M* KNO_3 and 0.1 m*M* $(NH_4)_6Mo_7O_{24}$, and in the presence or absence of 100 μ*M* $NaVO_4$ and 3 m*M* $MgSO_4$. PM-ATPase activity was measured at 38°C, representing the potential activity of the enzyme, and at 5, 12 or 20°C, representing the real activity at the actual root growth temperatures. The results are calculated in terms of vanadate-sensitive ATPase activity.

The purity of the PM fraction was determined by measuring the activities of cytochrome *c* oxidase (EC 1.9.3.1; inner mitochondrial membrane; Hodges and Leonard 1974), antimycin A-insensitive NADH cytochrome *c* reductase (EC 1.6.2.4; outer mitochondrial membrane, endoplasmic reticulum; Hodges and Leonard 1974), NO_3^--sensitive ATPase (EC 3.6.1.3; tonoplast; Briskin et al. 1987) and Triton X-100 stimulated UDPase (EC 3.6.1.6; Golgi apparatus; Nagahashi and Kane 1982). The substrate specificity and inhibitor sensitivity of the fraction were measured as described by Mattheis and Ketchie (1990). Membrane protein was determined according to Bradford (1976).

Fatty acids of phospholipids

Total lipids were extracted from the PM-enriched fractions according to Bligh and Dyer (1959). Phospholipids were separated and their fatty acids identified as described by Ryyppö et al. (1994). The fatty acids were converted to methyl esters and separated by GC (Carlo Erba Instruments, Milan, Italy; capillary column, fused silica 2-4018, Supelco, Bellefonte, PA, USA). Because of the small amount of plasma membranes left in each sample, two replicate samples, separately isolated, were pooled for each lipid extraction. Furthermore, the results of the same RZTs on days 0–1, 4–6 and 8–10 were combined to obtain three or four extractions of each treatment combination (RZT, day) for statistical analyses.

Chemicals

Cytochrome *c* and the substrates for the determination of PM purity, PEG 3350, MOPS, EGTA, PMSF and BHT, were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Dextran T-500 from Pharmacia Biotech (Uppsala, Sweden), and other chemicals from

Merck (Darmstadt, Germany) and BDH (Poole, Dorset, UK).

Statistical analyses

The effects of RZT on shoot weight and root volume were tested by analyses of covariance, in which the initial weight of the shoot or the initial volume of the roots was used as the covariate. The effects of RZT, duration of the treatment and their interaction on gas exchange, PM-ATPase activity, bond index and C18:2/16:0 ratio were tested by two-way analysis of variance. The pairwise differences between the treatment means were analyzed with Tukey's multiple range test. The statistic program used was SYSTAT (1992) for Windows v. 5 (Systat Inc., Evanston, IL, USA).

Results

Growth

The growth of roots and shoots during the 10-day experiment was dependent on root zone temperature ($P < 0.001$), and significant differences in root volume were also found between the RZTs (Fig. 1). Initial root volume as covariate was not significant ($P = 0.057$), but initial shoot weight was ($P = 0.001$). At the end of the experiment, the mean root volume increments at RZTs of 5, 12 and 20°C were 20, 130 and 290 mm^3, respectively. The mean fresh weight increment of the shoot was significantly lower at 5°C (0.19 g) than at 12°C (0.48 g) and 20°C (0.51 g) (Fig. 1).

Gas exchange

RZT had a significant effect on net photosynthesis ($P = 0.002$), transpiration ($P = 0.038$) and stomatal conductance ($P = 0.042$). Furthermore, net photosynthesis ($P < 0.001$), stomatal conductance ($P = 0.013$) and intercellular CO_2 concentration ($P < 0.001$) changed significantly during the 10-day experiment. Also the interaction between RZT and day of measurement affected net photosynthesis ($P = 0.049$), transpiration ($P = 0.024$) and stomatal conductance ($P = 0.026$) of the seedlings.

At the beginning of the experiment, air temperature was increased from 5 to 20°C whereas the RZT was either maintained at 5°C, or increased to 12 or 20°C. At that time net photosynthesis was low, while intercellular CO_2 concentration, transpiration and stomatal conductance were high (Tab. 1). Later, net photosynthesis increased, while transpiration, intercellular CO_2 concentration and stomatal conductance started to decrease at 5 and 12°C. At 20°C, net photosynthesis, stomatal conductance and transpiration increased, at the same time as intercellular CO_2 concentration decreased (Tab. 1). As the changes in gas exchange parameters were highly time-dependent, significant differences between the RZTs were found only on day 10. At that time net photosynthesis was significantly higher at 20°C than at lower RZTs, and stomatal conductance and transpiration were significantly higher at 20°C than at 5°C (Tab. 1).

Purity of the plasma membrane fraction

The PM fraction of the roots, obtained by the two-phase partitioning technique, contained 9% of the amount of membrane protein in the microsomal fraction (data not shown). The PM fraction had a high degree of purity with respect to the enzyme activities of mitochondria, non-specific phosphatases, ER, tonoplast and vacuole, in that 94–98% of the activity of these enzymes was found in the lower, Dextran-rich fraction (Tab. 2). The PM-ATPase activity (>90%) was inhibited by vanadate but only slightly affected by inhibitors of vacuolar (KNO_3) or mitochondrial (NaN_3) ATPase or by those of non-specific phosphatases [$(NH_4)_6Mo_7O_{24}$] (Tab. 3). Furthermore, PM-ATPase showed high substrate specificity for ATP, for which the other substrates tested were poor substitutes (Tab. 4).

PM-ATPase activity and fatty acids of phospholipids

The potential PM-ATPase activity (measured at 38°C) at days 1 and 4 was slightly higher at an RZT of 20°C than at 5 and 12°C, while at day 10 it was lowest at 20°C (Fig. 2). These differences were, however, not statistically significant. Real PM-ATPase activity, determined at 5, 12 or 20°C, was dependent on RZT ($P < 0.001$); the activity measured at 5°C being the lowest,

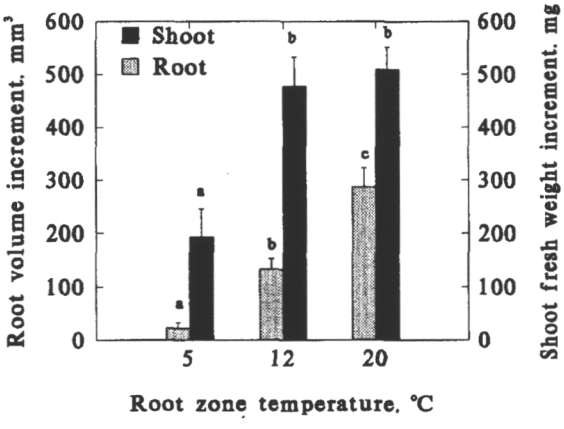

Fig. 1. Mean root volume and shoot weight increment (n = 40, vertical lines indicate +SE) of Scots pine seedlings exposed to root zone temperatures of 5, 12 or 20°C and shoot temperatures of 20/15°C (day/night). Within the root and shoot series, respectively, bars with a common letter are not significantly different at $P < 0.05$ when analyzed with Tukey's multiple range test.

Tab. 1. Gas exchange of Scots pine seedlings (n = 12, means ± SE) at the beginning of the second growth season. The roots of the seedlings were maintained at 5, 12 or 20°C during the 10-day experiment, while the shoots were at 20/15°C (18-h day/6-h night). Gas exchange was measured using a closed-system infrared gas analyzer for a period of 1 min after constant RH and a CO_2 concentration of about of 370 μl l^{-1} (22°C, RH 40–50% and photon flux density 550 μmol m^{-2} s^{-1}) was obtained. Gas exchange was calculated on the basis of measurements of shoot silhouette area. Values with a common letter are not statistically different ($P < 0.05$) when measurements of the same date are analyzed by Tukey's multiple range test.

Parameter	Root temperature		
	5°C	12°C	20°C
Net photosynthesis (μmol m^{-2} s^{-1})			
Day 1	1.7 ± 0.4a	2.6 ± 0.4a	1.7 ± 0.3a
Day 4	1.8 ± 0.6a	2.8 ± 0.5a	3.4 ± 0.5a
Day 10	2.6 ± 0.4a	3.6 ± 0.4a	5.0 ± 0.5b
Intercellular CO_2 (μl l^{-1})			
Day 1	306 ± 18a	280 ± 7a	291 ± 9a
Day 4	289 ± 28a	255 ± 6a	257 ± 11a
Day 10	182 ± 11a	200 ± 9a	205 ± 8a
Transpiration (mmol m^{-2} s^{-1})			
Day 1	0.96 ± 0.13a	0.96 ± 0.11a	0.83 ± 0.05a
Day 4	0.66 ± 0.07a	0.79 ± 0.12a	0.88 ± 0.07a
Day 10	0.48 ± 0.07a	0.71 ± 0.11ab	1.01 ± 0.11b
Stomatal conductance (mmol m^{-2} s^{-1})			
Day 1	65.5 ± 10.6a	70.4 ± 9.4a	55.2 ± 3.8a
Day 4	42.7 ± 4.8a	55.9 ± 10.2a	59.7 ± 5.2a
Day 10	26.8 ± 3.9a	43.4 ± 7.1ab	65.6 ± 7.9b

followed by the levels at 12 and 20°C (Fig. 3). The real PM-ATPase activity measured at 5 and 12°C did not change during the experiment, but that measured at 20°C decreased during the 10-day culture period (Fig. 3).

The most common fatty acid of the PM phospholipids in the roots of Scots pine seedlings was C18:2 (36–46%), followed by C16:0 (21–32%), C18:1 (15–21%) and by C18:3, C16:1 and C18:0 (3–8%), with a small fraction of C20:0 (1–2%) (Tab. 5). At an RZT of 5°C, the proportions of the fatty acids, and the C18:2/C16:0 ratio, as well as the bond index, remained almost unchanged during the 10-day experiment, whereas the proportions of C16:0 and C18:0 increased and those of C18:2 and C18:3 decreased at 12 and 20°C. Thus, the C18:2/C16:0 ratio and the bond index declined at 12 and 20°C (Tab. 5).

RZT and time had significant independent effects on the C18:2/C16:0 ratio ($P < 0.001$ and $P < 0.05$, respectively) and bond index ($P < 0.001$). Also the interaction between time and RZT affected the bond index ($P < 0.05$). As the changes in fatty acid saturation were time-dependent, statistically significant differences between the treatment means were found only on days 8–10 (Tab. 5). At that time, the proportions of C16:0, C18:0 and C18:1 were the highest and those of C16:1, C18:2 and C18:3 the lowest at 20°C. Thus, RZT affected the C18:2/C16:0 ratio and bond index at the end of the experiment, although initial changes were already to be seen on days 0–1 at 20°C and on days 4–6 at 12°C (Tab. 5).

Discussion

The increment in root volume of the Scots pine seedlings depended on RZT during the spring flushing (Fig. 1). Although both 5 and 12°C were suboptimal RZTs for root growth, the effect on shoot fresh weight

Tab. 2. Distribution of marker enzyme ([1]tonoplast, [2]endoplasmic reticulum and outer mitochondrial membrane, [3]inner mitochondrial membrane, [4]Golgi vesicles) activity after aqueous two-phase partitioning of the microsomal fraction of Scots pine root homogenates. Values represent the percentage of enzyme activity found in each phase in relation to the combined total from both phases. Six randomly selected samples, each with three replicate measurements, were used for the analyses.

Marker enzyme	Upper phase, % (PEG-rich)	Lower phase, % (Dextran-rich)
NO_3^--sensitive ATPase[1]	6	94
NADH cytochrome *c* reductase[2]	5	95
Cytochrome *c* oxidase[3]	3	97
Triton X-100 stimulated UDPase[4]	2	98

Tab. 3. Inhibitor sensitivity of PEG-rich upper phase ATPase activity after aqueous two-phase partitioning of the microsomal fraction of Scots pine roots. Inhibitors for [1]non-specific phosphatases, [2]mitochondrial ATPase, [3]tonoplast ATPase, [4]plasma membrane H^+-ATPase. Values are the mean specific activities ± SE of 6 samples, each with two replicate assays, without (control) or with inhibitor. Randomly selected different samples were used for each inhibitor.

Inhibitor	Specific ATPase activity, μmol (mg protein)$^{-1}$ min^{-1}		% of control
	Control	With inhibitor	
$(NH_4)_6Mo_7O_{24}$ (100 μ*M*)[1]	1.1 ± 0.3	1.2 ± 0.3	107
NaN_3 (1 m*M*)[2]	1.0 ± 0.2	0.9 ± 0.2	89
KNO_3 (50 m*M*)[3]	2.2 ± 0.4	2.0 ± 0.7	89
$NaVO_4$ (190 μ*M*)[4]	1.1 ± 0.2	0.1 ± 0.0	6

was observed only at 5°C, as earlier found for hydroponically cultured Scots pine seedlings (Ryyppö et al. 1994, Vapaavuori et al. 1992). A low RZT inhibits root growth by lengthening the duration of the cell division cycle (Barlow 1987) and by reducing the synthesis of growth substances in the roots (Atkin et al. 1973). Also, growth of the roots is determined by RZT, whereas both air and soil temperature can control the growth of the shoot (Larson 1967). A low RZT affects neither the timing of bud burst in Scots pine seedlings nor the duration of shoot sprouting in spring (Lyr and Garbe 1995, Vapaavuori et al. 1992), but it reduces the biomass of the current shoot (Ryyppö et al. 1994, Vapaavuori et al. 1992).

Gas exchange of the Scots pine seedlings was mainly dependent on the RZT (Tab. 1). The patterns of net photosynthesis, transpiration, stomatal conductance and intercellular CO_2 concentration showed that the RZT of 5°C in particular, but to some extent also that of 12°C, was suboptimal for photosynthesis, although shoot growth was affected only at 5°C. The low rate of net photosynthesis at 5 and 12°C, which restrained root growth, may indicate that allocation of photosynthates to the roots was limited by the low RZT, and that the supply of photosynthates was inadequate and did not allow optimum shoot growth at 5°C. Initially, root growth may be facilitated by carbohydrate reserves, but currently produced photosynthates are necessary for further elongation (Andersen et al. 1986). Thus, low RZT leads to competition for photosynthates between the root and shoot of northern pines, an effect which may be pronounced during the spring growth period (Eissenstat and Van Rees 1994).

Tab. 4. Substrate specificity of upper phase ATPase activity in the roots of Scots pine seedlings. Values are the mean specific activities ± SE from 6 assays, each including two replicate measurements, in the presence of 1 m*M* phosphorylated substrate; and the percentage of activity compared with a control having ATP as the substrate.

Substrate	Specific ATPase activity, μmol (mg protein)$^{-1}$ min^{-1}	% of control
ATP (control)	0.62 ± 0.11	100
ADP	0.20 ± 0.07	32
IDP	0.11 ± 0.01	17
UDP	0.10 ± 0.01	16
GTP	0.08 ± 0.02	13
UTP	0.05 ± 0.01	8
ITP	0.04 ± 0.02	6
AMP	0.03 ± 0.01	5
CTP	0.03 ± 0.00	5

At the beginning of the experiment, net photosynthesis of the seedlings was primarily limited by non-stomatal factors (Tab. 1). At that time, high intercellular CO_2 concentration, transpiration and stomatal conductance indicated that the high mesophyll resistance lowered the rate of carboxylation (DeLucia and Smith 1987). During the recovery from winter inhibition of photosynthesis (Strand and Lundmark 1995) net photosynthesis increased and led to a controlled gas exchange pattern, which was dependent on RZT. Due to increasing stomatal conductance, the rates of net photosynthesis and transpiration increased at 20°C, causing the decrease of the intercellular CO_2 concentration on days 4 and 10. The uptake of water at this RZT was then facilitated by the rapid growth of new roots (Fig. 1), although the temperature-induced changes in PM structure and function may also lower the resistance to water flow in the roots (Fig. 3, Tab. 5). At 5 and 12°C, transpiration was reduced by lowering stomatal conductance on days 4 and 10, which indicates that the low RZT became the main limiting factor for net photosynthesis (DeLucia and Smith 1987). Low RZT affects the transfer of water to roots because of the higher viscosity of water, lower permeability of root cell membranes, smaller amount of unsuberized roots, and reduced metabolic activity in the roots (DeLucia et al. 1991, Häussling et al. 1988, Kramer 1983). Furthermore, a difference in temperatures between the root and shoot (5/20°C and 12/20°C, respectively), which is a common day-time phenomenon in boreal forests in spring, may lead to an imbalance between water uptake and transpiration and cause a water deficit in the elongating shoot (Lopushinsky and Kaufmann 1984, Vapaavuori et al. 1992).

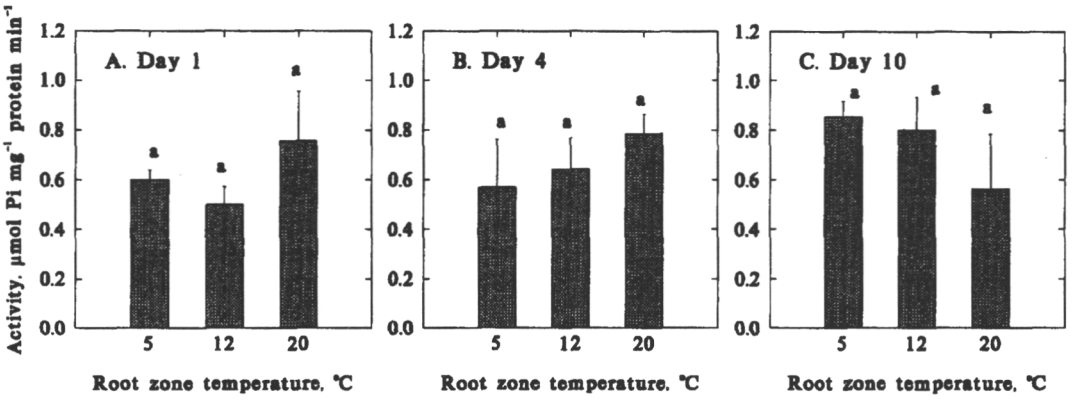

Fig. 2. Potential plasma membrane H^+-ATPase activity (measured at 38°C) on day 1 (A), day 4 (B) and day 10 (C). The roots of Scots pine seedlings were exposed to root zone temperatures of 5, 12 or 20°C and shoots to temperatures of 20/15°C (day/night). Vertical lines indicate +SE (n = 4). Bars carrying the same letter are not significantly different ($P < 0.05$) when the samples of the same day are analyzed with Tukey's multiple range test.

Saturation of phospholipid fatty acids in the plasma membrane of Scots pine roots was dependent on RZT (Tab. 5), as previously observed in a microsomal fraction of root cell membranes (Ryyppö et al. 1994). The degree of phospholipid fatty acid saturation in the aerial parts of cold hardening species decreases in autumn (Sutinen 1992, Yoshida 1984), and these changes are reversed during deacclimation in spring (Sutinen 1992, Yoshida 1986). RZT of 5°C, which inhibited root growth and retarded shoot growth, also restrained the saturation of PM phospholipids in the roots. At the higher RZTs the degree of fatty acid saturation increased, mainly on account of the decline in the C18:2/C16:0 ratio in the course of the experiment (Tab. 5).

Changes in the degree of phospholipid fatty acid saturation in the PM, and especially the change in the C18:2/C16:0 ratio, are connected to frost hardening and dehardening of pine (Palta et al. 1993, Sutinen 1992). In our study, the RZT of 20°C caused a small increase in the degree of saturation and a decrease of the C18:2/C16:0 ratio one day after transfer of the seedlings to the experimental conditions, which may indicate a rapid dehardening process in the roots (Palta et al. 1993, Sutinen 1992). At 12°C these changes occurred later, after 4–6 days, which indicates

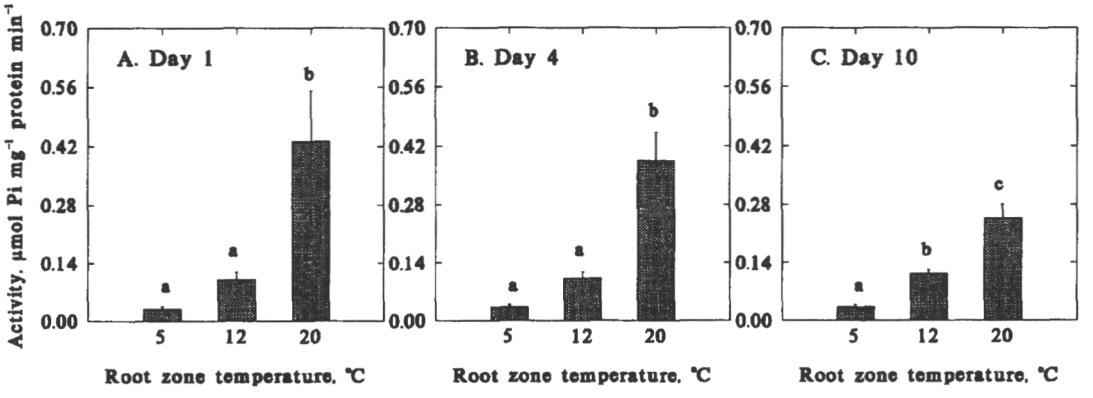

Fig. 3. Plasma membrane H^+-ATPase activity of Scots pine roots measured on day 1 (A), day 4 (B) and day 10 (C) at the real root zone temperatures of 5, 12 or 20°C. Vertical lines indicate +SE (n = 4). Bars carrying the same letter are not significantly different at $P < 0.05$ when samples of the same day are analyzed with Tukey's multiple range test.

Tab. 5. Changes in fatty acids of plasma membrane phospholipids and in the bond index (C16:1 + C18:1 + 2 × C18:2 + 3 × C18:3) in the roots of Scots pine seedlings subjected to root temperatures of either 5, 12 or 20°C. The results are means of three or four preparations (±SE) made on days 0 and 1 (0–1), 4 and 6 (4–6), and 8 and 10 (8–10). Each preparation was pooled from two separately isolated plasma membrane samples. Values with a common letter are not statistically different at $P < 0.05$ when measurements made on the same day are analyzed by Tukey's multiple range test.

Day	Root temperature, °C	Fatty acids of phospholipids, % of total						C18:2/C16:0	Bond index
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	
0–1	5	22.3 ± 0.7a	4.30 ± 1.08a	4.17 ± 0.21a	17.1 ± 1.1a	44.5 ± 1.5a	6.36 ± 0.30a	1.18 ± 0.03a	2.00 ± 0.08a	130 ± 2a
	12	20.8 ± 1.4a	3.76 ± 0.22a	3.83 ± 0.26a	21.1 ± 2.2a	42.1 ± 2.4a	7.87 ± 0.29a	1.45 ± 0.26a	2.05 ± 0.24a	130 ± 4a
	20	24.1 ± 0.8a	4.64 ± 0.18a	3.87 ± 0.46a	18.9 ± 2.8a	39.3 ± 1.4a	8.80 ± 1.23a	1.36 ± 0.09a	1.63 ± 0.01a	126 ± 4a
4–6	5	23.1 ± 0.6a	4.04 ± 0.33a	4.02 ± 0.85a	14.5 ± 0.8a	45.6 ± 1.4a	7.40 ± 0.41a	1.30 ± 0.09a	1.98 ± 0.11a	132 ± 3a
	12	25.3 ± 0.7a	5.96 ± 0.57a	3.43 ± 0.29a	16.6 ± 0.7a	40.4 ± 1.1a	7.01 ± 0.46a	1.36 ± 0.28a	1.60 ± 0.07a	124 ± 2a
	20	23.1 ± 0.7a	6.20 ± 1.65a	4.84 ± 0.53a	21.0 ± 3.5a	36.0 ± 5.7a	7.14 ± 0.88a	1.63 ± 0.25a	1.55 ± 0.21a	120 ± 5a
8–10	5	22.1 ± 0.9a	5.15 ± 1.14a	5.22 ± 0.15ab	15.8 ± 0.2a	42.8 ± 2.2a	6.95 ± 0.27a	1.80 ± 0.47a	1.95 ± 0.17a	128 ± 4a
	12	25.8 ± 2.0a	5.10 ± 0.89a	4.72 ± 0.82a	19.2 ± 3.3a	38.5 ± 2.1ab	5.33 ± 0.41b	1.32 ± 0.06a	1.50 ± 0.08a	117 ± 3a
	20	31.6 ± 0.6b	3.30 ± 0.45a	7.37 ± 0.59b	19.6 ± 0.6a	32.6 ± 1.2b	3.54 ± 0.36c	1.90 ± 0.33a	1.03 ± 0.06a	99 ± 3b

that the rate of root dehardening was dependent on RZT. In the course of the experiment, the proportion of C18:3 in the pine roots decreased at 12 and 20°C and at the end of the study the proportion remained significantly higher at 5°C than at 12 or 20°C (Tab. 5). The opposite response, an increment in the proportion of C18:3, takes place in the aerial parts during deacclimation (Palta et al. 1993).

The measurements of PM-ATPase activities at real RZTs (5, 12 or 20°C) as compared to those at 38°C, showed that the catalytic activity of the enzyme was strongly limited by low temperature (Figs 2 and 3). An increase in temperature caused activation of PM-ATPase, which can be concluded from the high potential activity (measured at 38°C) compared with the activity measured at real RZT from the same sample. The potential and real PM-ATPase activity (measured at 38 and 20°C, respectively) decreased in roots maintained at 20°C (Figs 2 and 3). In Scots pine needles such a decrease in the PM-ATPase activity, as well as the observed saturation of PM phospholipids, is connected to dehardening (Hellergren et al. 1983, Sutinen 1992). The modulation of phospholipid fatty acids may alter the enzyme activity in the membranes (Cooke and Burden 1990, Palmgren et al. 1988) and, in particular, changes in the C18:2/C16:0 ratio in PM phospholipids may regulate PM-ATPase activity (Palmgren et al. 1988, Sutinen 1992). If no changes in C18:2/C16:0 ratio occur, as observed at 5°C in the present study, the potential PM-ATPase activity may even increase (Fig. 2), indicating delayed dehardening of roots.

We conclude that low RZT delays the structural and functional changes in PM of Scots pine roots, such as saturation of PM phospholipids, increase in real activity and decrease in potential PM-ATPase activity, which may be connected to dehardening in spring. Low RZT inhibits root growth and causes an imbalance between water uptake and transpiration, leading to water shortage in the shoot, lower rate of net photosynthesis and decreased availability of photosynthates for growth. A rise in RZT induces fast changes in the saturation of PM phospholipids, and especially reduces the C18:2/C16:0 ratio. These changes may modulate PM-ATPase activity and fasten root growth, allowing efficient uptake of water and nutrients from the root medium. Such alterations may be crucial for fast and successful acclimation of Scots pine seedlings to a new planting site in spring.

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II

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Responses of Scots pine (*Pinus sylvestris*) seedlings grown in different nutrient regimes to changing root zone temperature in spring

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Summary We examined effects of nutrient availability and changing root zone temperature (RZT) on growth, gas exchange and plasma membrane H^+-ATPase (PM-ATPase) activity of roots of 1-year-old Scots pine (*Pinus sylvestris* L.) seedlings during spring flushing. The 6-week growth-chamber experiment was carried out in hydroponic cultures that supplied the seedlings with low (0.5 mM N) or high (3 mM N) nutrient concentration and two rates of increase in RZT were simulated: slow warming (SW-treatment) and fast warming (FW-treatment). Air temperature, humidity, and light conditions were similar in all treatments. Growth of roots and shoots was retarded at low RZT, and fresh mass increment of roots was closely correlated with RZT sum. High nutrient availability increased nitrogen concentrations of needles and stems, but only at RZTs >13 °C. Low RZT and low availability of nutrients suppressed gas exchange of the seedlings . Real PM-ATPase activity was highly dependent on RZT. At high RZTs, real PM-ATPase activity was affected by nutrient availability but this effect was related to root growth. We conclude that, under conditions of high nutrient availability, Scots pine seedlings can compensate for the suppressive effects of long-term exposure to low RZT by rapidly accelerating growth, gas exchange and root metabolism, but only when RZT has increased above a threshold value, which was 13 °C in this study.

Keywords: gas exchange, growth, H^+-ATPase, nitrogen, nutrient availability, plasma membrane, roots.

Introduction

In boreal forests, conifer seedlings are usually planted in early spring before or during bud burst. An important event affecting the further survival of the seedlings is the formation of good root-soil contact, which is necessary for water and nutrient uptake (Burdett 1990). However, at the time of planting, soil temperature is usually much lower than air temperature and warming of the soil occurs slowly, especially when the soil is deeply frozen. Low soil temperatures may seriously retard establishment of newly transplanted seedlings. Low, nonfreezing root zone temperatures (RZT) can delay both root and shoot growth (Lopushinsky and Kaufmann 1984, Vapaavuori et al. 1992, Ryyppö et al. 1994, 1998) and decrease shoot gas exchange (DeLucia 1986, Ryyppö et al. 1994, 1998) as well as water (Lopushinsky and Kaufmann 1984) and nutrient (Kramer 1983) uptake.

Previously, we showed that plasma membrane H^+-ATPase (PM-ATPase) activity of roots is limited at low RZTs (Ryyppö et al. 1994, 1998). Root PM-ATPase is involved in several physiological processes including nutrient uptake, regulation of intracellular pH, cell wall extension, and various turgor-related phenomena (reviewed by Michelet and Boutry 1995). Transport of many solutes (ions, metabolites, etc.) in and out of the root cell involves secondary transporters whose function PM-ATPase energizes directly by creating an electrochemical gradient between the outer and inner side of the plasma membrane (Michelet and Boutry 1995).

Solute flow through the roots is also affected by nutrient deprivation, as a result of decreased root hydraulic conductance (Chapin et al. 1988, Radin 1990, Carvajal et al. 1996). According to Carvajal et al. (1996) nutrient deprivation can decrease hydraulic conductance of wheat roots by changing both lipid composition of the plasma membrane and membrane fluidity. Changes in lipid composition can also modulate PM-ATPase activity (Sandstrom and Cleland 1989, Cooke and Burden 1990). Thus, it is possible that nutrient deprivation also affects PM-ATPase activity directly by modulating lipid composition of the plasma membrane.

Photosynthetic capacity of leaves is related to their nitrogen concentration (Evans 1989). The efficiency of gas exchange, which is affected by water and nutrient availability, affects not only shoot growth but also root growth because a supply of current photosynthates from the shoot seems to be necessary for root elongation in conifer seedlings (Andersen et al. 1986, Eissenstat and Van Rees 1994). Thus, nutrient availability from the soil may affect growth initiation of the seedlings during spring flushing.

Previously, we focused on responses of Scots pine (*Pinus sylvestris* L.) seedlings to various but constant RZTs during growth initiation in spring (Vapaavuori et al. 1992, Ryyppö et al. 1994, 1998). However, such temperature conditions do not occur in nature, because soil temperature generally follows with a delay the changes in air temperature. Therefore, we

have simulated more "natural" RZT conditions, slow warming and fast warming, to mimic deep-frost and no-frost conditions in spring. To test the hypothesis that retardation of root growth and physiological performance by low RZT can be affected by increasing nutrient availability, we examined the interaction of RZT and nutrient availability on growth and gas exchange of Scots pine seedlings.

Materials and methods

Plant material

The experiment was conducted with one-year-old Scots pine (*Pinus sylvestris* L.) seedlings grown in peat-filled paper pots at the nursery of Suonenjoki Research Station. The seed originated from a seed orchard in Central Finland (62°05′ N, 26°10′ E) that has been established with seed from different sites located in Finland between 61°70′ N and 64°20′ N. In mid-October, the seedlings were transferred to cold storage and kept at −5 °C over winter. At the beginning of April 1996, the seedlings were thawed in darkness at 4 °C for 7 days. Peat was removed from the roots of the thawed seedlings by gentle washing with cold tap water. After washing, 192 seedlings were randomly sampled (Day 0 samples). Thereafter, 1152 seedlings were placed in 40-dm^3 plastic containers (96 seedlings per container) filled with aerated nutrient solution containing a low concentration of nutrients (0.5 mmol N dm^{-3}) (Ingestad and Lund 1986).

Experimental design

The containers with the seedlings were placed in a growth chamber (Weiss, type 10 Sp/5 DU-Pi, Lindenstruth, Germany). For the first three days, the seedlings were kept continuously in dim light (125 µmol m^{-2} s^{-1}), and air and root temperatures were maintained at 4 °C. After three days, the experiment was conducted in a factorial manner. The containers were first grouped into two RZT treatments, slow warming (SW-treatment) and fast warming (FW-treatment), each having six containers. In the SW-treatment, RZT increased from 4 to 6 °C during Days 0–14, from 6 to 13 °C during Days 15–28 and from 13 to 18 °C during Days 29–42. In the FW-treatment, RZT increased from 4 to 14 °C during Days 0–14, from 14 to 18 °C during Days 15–28 and remained at 18 °C during Days 29–42. Containers were further divided into high-nutrient (3 mmol N dm^{-3}; HN-treatment) and low-nutrient (0.5 mmol N dm^{-3}; LN-treatment) treatments. Both nutrient solutions (Ingestad and Lund 1986) contained nitrate and ammonium ions in the same proportion, 58 and 42% of the total nitrogen, respectively. Concentrations of other nutrients were proportional to the nitrogen concentration. The pH of the nutrient solutions was adjusted to 4.5–5.5 by adding 1 N NaOH or 1 N HCl. The concentrations of the nutrient solutions were kept constant throughout the 6-week experiment. The daily fluctuation in air temperature was similar in all treatments (Figure 1A). The RZTs were controlled by circulating the nutrient solution through cryostats (MGW, Lauda, Germany) (Figure 2A)

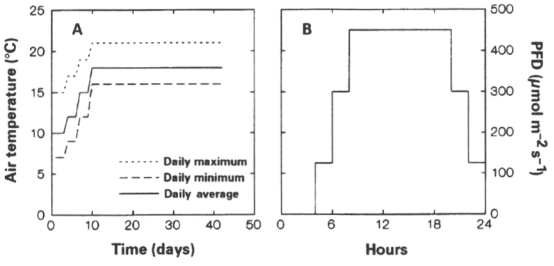

Figure 1. Average, minimum and maximum daily air temperatures (A) and daily fluctuation in photon flux density (B) in the growth chamber during the 6-week experiment.

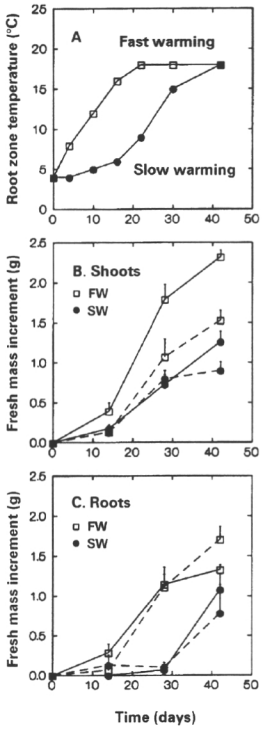

Figure 2. Root zone temperature (RZT) (A), and mean fresh mass increment of shoots (B) and roots (C) of Scots pine (*Pinus sylvestris* L.) seedlings exposed to slow (SW) or fast warming (FW) of the root zone and grown at high (3 mM N) or low (0.5 mM N) nutrient availability during the 6-week experiment. Solid line = HN-treatment (3 mM N) and broken line = LN-treatment (0.5 mM N). Data are means (± SE) of 30 replicates.

and were measured daily with a digital thermometer (Sensotherm 100, Sensotherm, Nürnberg, Germany). Day/night relative air humidity was 45/70% and the photoperiod was 20 h. Chamber air temperature and relative humidity were continuously recorded by a thermohygrograph. Maximum daily photon flux density at the top of the seedlings was

450 μmol m^{-2} s^{-1} (PAR, LI-185B, Li-Cor, Inc., Lincoln, NE) (Figure 1B).

Growth analysis

Root volume was determined by the displacement method (Burdett 1979), which allows repeated measurements of the same seedlings. Measured root volume was assumed to equal the fresh mass of roots in hydroponic culture. Shoot fresh mass was determined by subtracting root mass from whole-seedling mass.

Growth was determined by weighing ten seedlings per container at the beginning of the experiment and 14, 28 and 42 days later. The initial fresh mass of roots was 2.05 ± 0.66 g (± SD, n = 120), and the initial fresh mass of shoots was 2.58 ± 0.55 g (n = 120). Relative growth rates (RGR) of shoots and roots were calculated as $RGR = (\ln W_2 - \ln W_1)/(t_2 - t_1)$ (Ingestad 1982), where W_2 and W_1 represent the fresh mass of shoots and roots at sampling times t_2 and t_1, respectively.

Gas exchange measurements

Gas exchange of the whole shoot (12 seedlings per treatment) was determined by repeated measurements with a closed-system infrared gas analyzer (LI-6200, Li-Cor, Inc.) 22 and 42 days after the start of the experiment. During measurements, RZT was maintained at the same temperature as the treatment temperature, shoot temperature was 22 °C and photon flux density was 600 μmol m^{-2} s^{-1} PAR (cool-beam halogen lamps FTY GX5.3, Sylvania, Belgium). Gas exchange was measured for 1 min after a constant relative humidity of 40–50% and a CO_2 concentration of 370 μl l^{-1} had been established. Gas exchange rates were calculated on the basis of shoot silhouette area, which was measured with a video camera connected to a graphic monitor.

Determination of total nitrogen

On Days 0, 10, 22 and 42, seedlings were sampled for total nitrogen determinations. Altogether 16 seedlings were sampled systematically from each container and the shoots were separated into stems and needles. The fresh masses of pooled stems and needles were determined and the plant parts were then dried at 60 °C for 48 h and weighed. Total nitrogen of the pooled samples was measured with a CHN-600 Analyzer (Leco Co., St. Joseph, MI).

Isolation of plasma membranes from roots

Material for plasma membrane isolation was sampled on Days 0, 4, 10, 16, 22, 30 and 42. Sixteen seedlings were sampled systematically from each container and the roots were excised. The excised roots from each container were pooled, frozen in liquid nitrogen and stored at –80 °C.

Plasma membranes were isolated by a two-phase aqueous polymer technique (Widell 1987). About 30–40 g of frozen roots were ground with a pestle in a mortar filled with liquid nitrogen. The finely ground roots were thawed by stirring in 100 ml of ice-cold homogenizing buffer pH 7.3 (Uemura and Yoshida 1983) containing 0.5 M sucrose, 75 mM MOPS-NaOH, 5 mM EGTA, 1 mM PMSF in isopropanol, 2 mM salicyl hydroxamic acid, 2.5 mM sodium bisulfite, 1.5% (w/v) PVP (M_r 24,000), 0.5 % (w/v) defatted BSA and 10 mg l^{-1} BHT. The ground roots were further homogenized with a Polytron PT 3000 homogenizer at 13,000 rpm for 3 × 20 s in an ice bath. To remove starch grains, nuclei, unbroken plastids and mitochondria, the homogenate was filtered twice through cheesecloth and centrifuged at 8500 g for 20 min at 4 °C. The supernatant was centrifuged at 48,000 g for 2 h at 4 °C to yield a pellet containing microsomal membranes. The pellet was resuspended in 10 ml of ice-cold sucrose-phosphate buffer (0.25 M sucrose in 10 mM K-phosphate buffer, 0.2 mM PMSF, 10 mg l^{-1} BHT, and 1mM DTT, pH 7.3). An aliquot (4.5 ml) of the suspension was added to an aqueous two-phase mixture producing a 36-g two-phase system with a final composition of 5.7% Dextran T-500 (Pharmacia Biotech), 5.7% polyethylene glycol 3500, 0.3 M NaCl and 0.25 M sucrose-phosphate buffer. The phases were separated by centrifugation in a swing-out rotor at 1500 g for 15 min at 4 °C. The separated upper phases were collected and diluted with a phosphate-free buffer (0.53 M sucrose, 5 mM MOPS-NaOH, 1 mM EGTA, 10 mM KCl, 0.2 mM PMSF, 10 mg l^{-1} BHT and 1 mM DTT, pH 7.3) and centrifuged at 48,000 g for 2 h at 4 °C. The resulting pellets were suspended in phosphate-free buffer and stored at –80 °C. This isolation procedure produces a highly pure plasma membrane fraction from Scots pine roots (Ryyppö et al. 1998).

Assay of PM-ATPase activity and determination of membrane proteins

Activity of PM-ATPase was measured as described by Ryyppö et al. (1994) with slight modifications. The assay is based on colorimetric measurement of the amount of Pi released from ATP hydrolysis (Hodges and Leonard 1974). Activity was assayed in the presence of 5 mM NaN_3, 100 mM KNO_3 and 0.1 mM $(NH_4)_6Mo_7O_{24}$, and in the presence or absence of 100 mM $NaVO_4$ and 3 mM $MgSO_4$. The detergent used was 0.1% Brij 58. Activity of PM-ATPase was measured at the actual RZT and at 38 °C. Vanadate-sensitive PM-ATPase activity was calculated based on the amount of plasma membrane proteins. The activity measured at the actual RZT is a measure of the enzyme activity at real RZT and hereafter is referred to as "the real PM-ATPase activity." The activity measured at 38 °C shows the maximal capacity of the enzyme and hereafter is called "the potential PM-ATPase activity." The amount of membrane protein was estimated according to Bradford (1976).

Statistical analyses

Effects of rate of increase in RZT and nutrient availability on fresh mass increment of shoot and roots were tested by three-way analysis of covariance with temperature treatment (SW- and FW-treatment), nutrient treatment (LN- and HN-treatment) and duration of treatments as factors. The initial fresh mass of shoot or roots was used as a covariate. Mean values for fresh mass of roots and shoots of ten seedlings in each container were used as independent observations in the statistical analysis. Three-way analysis of variance was used with

temperature treatment, nutrient treatment and duration of treatment as factors when the effects of rate of increase in RZT and nutrient availability on gas exchange, nitrogen concentration, PM-ATPase activity, root/shoot ratio and relative growth rate (RGR) were tested. Values for nitrogen concentrations, PM-ATPase activities, root/shoot ratios and RGR of the pooled samples for each container were used as independent observations. When gas exchange was measured, each seedling was used as an independent observation. We used the statistical program SYSTAT (1992) for Windows 5.0.

Results

Root and shoot development

Shoot growth was significantly affected by rate of RZT increment ($P < 0.001$) and duration of treatment ($P < 0.001$). There was also a significant interaction between rate of RZT increment and duration of treatment ($P = 0.005$). Initial fresh mass of the shoot as a covariate was not statistically significant. Shoot fresh mass increment was higher in seedlings in the FW-treatment than in the SW-treatment (Figure 2B). Shoot growth was significantly affected by nutrient availability ($P < 0.001$). In HN-treatments, shoot growth was accelerated at RZTs above 13 °C. Shoot RGR was affected by RZT, nutrient availability and duration of treatment ($P = 0.002$, 0.041 and < 0.001, respectively). In all treatments, shoot RGR was higher during Days 15–28 than during Days 29–42 (Table 1).

Rate of RZT increment and duration of treatment significantly affected root growth ($P < 0.001$ for both). There was also a significant ($P = 0.003$) interaction between rate of RZT increment and duration of treatment. Initial fresh mass of roots as a covariate was not statistically significant. In the SW-treatment, root growth was slow until RZT exceeded 13 °C (Figure 2C). Root flushing started earlier and root mass increment was higher in seedlings in the FW-treatment than in the SW-treatment. Root RGR was significantly affected by duration of treatment ($P < 0.001$), and there was also a significant interaction between RZT and duration of treatment ($P < 0.001$). In seedlings in the SW-treatment, root RGR was low during Days 1–28, but increased markedly during Days 29–42 (Table 1). In contrast, in seedlings in the FW-treatment, root RGR was highest during Days 15–28.

Nutrient availability affected the allocation of growth between shoots and roots more when RZT was increased rapidly than when RZT was increased slowly ($P = 0.023$) (Table 2). The root/shoot ratio decreased in the HN-treatment and increased in the LN-treatment in response to fast warming.

To determine whether the slow rise in RZT suppressed root growth, fresh mass increment of the roots was plotted against the effective temperature sum (d.d.) of the root zone (d.d. = degree days above a threshold 5 °C, Figure 3B). The RZT sum was 229 d.d. in the SW-treatment at the end of the experiment (Day 42), whereas in the FW-treatment the same temperature sum was obtained on Day 27. The fresh mass increment of the roots was closely correlated with RZT sum, regardless of the rate of RZT increment (Figure 3B). In contrast, the fresh mass increment of the shoot was not affected by the RZT sum (Figure 3A), but it was affected by the effective air temperature sum, which developed in the same way in all the treatments (data not shown).

Gas exchange

Rate of RZT increment, nutrient availability, and duration of treatment all significantly affected net photosynthesis, transpiration and stomatal conductance of the seedlings ($P < 0.001$) (Table 3). Intercellular CO_2 concentration showed a significant response to rate of RZT increment ($P = 0.011$) and nutrient availability ($P = 0.038$). Low RZT and low nutrient availability suppressed gas exchange; and the highest values for net photosynthesis, transpiration and stomatal conductance were observed in the FW + HN-treatment. There were also differences in gas exchange parameters between the nutrient treatments in the SW-treatment on Day 22 when RZT was only 9 °C.

Nitrogen concentrations of the needles and stem

Needle nitrogen concentration was significantly affected by rate of RZT increment ($P < 0.01$), nutrient availability ($P < 0.001$) and duration of treatment ($P < 0.001$). The interactions between rate of RZT increment and duration of treatment and between nutrient availability and duration of the treatment were significant ($P < 0.001$ for both). Needle nitrogen concentration decreased with time in the LN + SW and LN + FW treatments (Figures 4A and 4C). In the SW + HN treatment, needle nitrogen concentration decreased during the first

Table1. Effects of rate of RZT increment and nutrient availability on the relative growth rates (RGR) of roots and shoots of Scots pine seedlings (*Pinus sylvestris*) during the 42-day experiment. The RGR is given as the relative daily increase in shoot and root fresh mass (mg g^{-1} day^{-1}) ($n = 30$, mean ± SE). When the rate of RZT increment was slow, RZT increased from 4 to 6 °C during Days 0–14, from 6 to 13 °C during Days 15–28 and from 13 to 18 °C during Days 29–42. When the rate of RZT increment was fast, RZT increased from 4 to 14 °C during Days 0–14, from 14 to 18 °C during Days 15–28 and remained at 18 °C during Days 29–42. Abbreviations: HN = high nutrient and LN = low nutrient.

Days of treatment	Slow warming (SW-treatment)				Fast warming (FW-treatment)			
	RGR in HN-treatment		RGR in LN-treatment		RGR in HN-treatment		RGR in LN-treatment	
	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots
1–14	0 ± 0	4.8 ± 1.4	4.2 ± 4.2	3.1 ± 1.6	9.5 ± 3.7	10.3 ± 2.4	2.3 ± 2.3	3.6 ± 1.8
15–28	4.3 ± 2.2	13.1 ± 1.9	2.4 ± 2.4	16.0 ± 1.9	22.5 ± 4.7	27.9 ± 2.3	31.1 ± 9.4	22.1 ± 5.8
29–42	31.7 ± 4.9	10.7 ± 1.0	21.2 ± 2.7	2.1 ± 0.9	5.4 ± 2.7	8.5 ± 2.8	12.7 ± 3.1	8.6 ± 2.2

Table 2. Root/shoot fresh mass ratio of Scots pine (*Pinus sylvestris*) seedlings sampled during the 42-day experiment ($n = 30$; values are means ± SE). Abbreviations: HN = high nutrient, LN = low nutrient, and RZT = root zone temperature.

Days of treatment	Slow warming (SW-treatment)			Fast warming (FW-treatment)		
	RZT, °C	HN-treatment	LN-treatment	RZT, °C	HN-treatment	LN-treatment
0	4	0.77 ± 0.05	0.81 ± 0.04	4	0.81 ± 0.02	0.80 ± 0.04
14	6	0.69 ± 0.04	0.82 ± 0.12	14	0.79 ± 0.07	0.76 ± 0.11
28	13	0.60 ± 0.02	0.63 ± 0.02	18	0.74 ± 0.06	0.87 ± 0.03
42	18	0.80 ± 0.04	0.82 ± 0.01	18	0.70 ± 0.04	0.92 ± 0.03

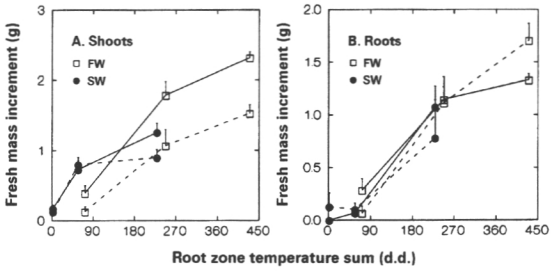

Figure 3. Mean fresh mass increment of shoots (A) and roots (B) of Scots pine (*Pinus sylvestris*) seedlings versus root zone temperature sum in degree days (d.d.). The seedlings were exposed to slow (SW) or fast warming (FW) of the root zone and grown at high (3 mM N, solid line) or low (0.5 mM N, broken line) nutrient availability. Data are means (± SE) of 30 replicates.

22 days but increased when RZT increased from 9 to 18 °C during Days 23–42 (Figure 4A). In the FW + HN treatment, needle nitrogen concentration remained constant during the first 10 days and increased at the end of the experiment (Figure 4C).

Nitrogen concentration of the stem was significantly affected by nutrient availability ($P < 0.001$) and duration of treatment ($P < 0.001$). In addition, the interaction between nutrient availability and duration of treatment was significant ($P < 0.001$). Nitrogen concentration of the stem decreased with time in all treatments, but was higher in HN-treatments than in LN-treatments at the end of the experiment (Figures 4B and 4D).

Plasma membrane PM-ATPase activity of the roots

Real PM-ATPase activity was significantly affected by rate of RZT increment ($P = 0.001$) and duration of treatment ($P < 0.001$), but not by nutrient availability ($P = 0.052$) (Figures 5B and 5E). In addition, the interaction between rate of RZT increment and duration of treatment on real activity was significant ($P < 0.001$) (Figures 5B and 5E).

In the SW-treatment, real activity was low until RZT was 9 °C (Figure 5B). At the end of the experiment, when RZT had increased to 18 °C, real PM-ATPase activity was strongly affected by nutrient availability, being almost twice as high in the HN-treatment compared with the LN-treatment. In the FW-treatment, real PM-ATPase activity increased in parallel with increasing RZT until RZT exceeded 18 °C (Figure 5E). During the last 20 days when RZT had reached a temperature of 18 °C, real PM-ATPase activity decreased. The reduction in real PM-ATPase activity occurred earlier in the LN-treatment

Table 3. Gas exchange of Scots pine (*Pinus sylvestris*) seedlings 22 and 37 days after the start of the experiment ($n = 12$, values are means ± SE). At Day 22, RZT was 9 °C in the SW-treatment and 18 °C in the FW-treatment. At Day 37, RZT was 18 °C in all treatments. Abbreviations: HN = high nutrient and LN = low nutrient.

Parameter	Slow warming (SW-treatment)		Fast warming (FW-treatment)	
	HN-treatment	LN-treatment	HN-treatment	LN-treatment
Net photosynthesis μmol m^{-2} s^{-1}				
Day 22	5.9 ± 0.3	4.0 ± 0.4	7.8 ± 0.3	6.4 ± 0.4
Day 37	9.4 ± 0.5	7.3 ± 0.9	12.7 ± 0.7	9.3 ± 0.5
Intercellular CO_2 μl l^{-1}				
Day 22	187.6 ± 5.4	203.5 ± 8.1	204.1 ± 4.4	211.2 ± 3.0
Day 37	207.8 ± 5.2	199.0 ± 6.2	202.1 ± 4.6	219.6 ± 3.0
Transpiration mmol m^{-2} s^{-1}				
Day 22	1.4 ± 0.1	1.1 ± 0.1	2.1 ± 0.1	1.9 ± 0.1
Day 37	2.6 ± 0.2	2.0 ± 0.3	3.2 ± 0.2	2.7 ± 0.2
Stomatal conductance mmol m^{-2} s^{-1}				
Day 22	66.5 ± 5.6	49.5 ± 5.2	102.7 ± 6.9	87.3 ± 6.9
Day 37	130.1 ± 10.6	95.1 ± 14.9	172.5 ± 12.5	139.8 ± 9.5

than in the HN-treatment, but the reduction was smaller in the LN-treatment than in the HN-treatment.

Potential PM-ATPase activity demonstrated a more variable pattern than real PM-ATPase activity (Figures 5C and 5F). Potential PM-ATPase activity was not significantly affected by rate of RZT increment, nutrient availability or duration of treatment.

Discussion

Root growth of Scots pine seedlings was more sensitive to low RZT than shoot growth (Figures 2B and 2C). Reduced root growth at low RZTs has previously been reported for conifers (Lopushinsky and Kaufmann 1984, Lopushinsky and Max 1990, Vapaavuori et al. 1992, Ryyppö et al. 1994, 1998). Root growth accelerated when RZT increased above 13 °C (Table 1). In all treatments, root growth depended strongly on the RZT sum (Figure 3B). In contrast to root growth, shoot growth is affected more by air temperature than by soil temperature. Similarly, in several conifers, low RZT reduces biomass production of the current shoot, but timing of bud break and the start of shoot elongation are not greatly influenced by RZT (Lopushinsky and Kaufmann 1984, Vapaavuori et al. 1992, Lyr and Garbe 1995).

Nutrient availability had no effect on fresh mass increment of the shoot at low RZTs (4–13 °C in SW-treatment), indicating that inhibition of shoot growth at low RZTs is probably a consequence of low temperature rather than low nutrient availability. This supposition is supported by the finding that nitrogen concentrations of needles and stem were not affected by nutrient availability at low RZTs. When RZT was above 13 °C, both shoot and root growth accelerated rapidly in the HN-treatment, whereas only root growth was accelerated in the LN-treatment. Thus, at low RZTs, the balance between shoot and root growth is determined primarily by temperature, whereas at higher RZTs the balance is determined by the external supply of nutrients (Macduff et al. 1994). The effect of RZT and nutrient availability on growth and nitrogen concentration of the shoot suggest that initiation of growth in Scots pine seedlings only becomes dependent on high nutrient availability after a threshold RZT is reached. In this study the threshold was 13 °C.

Inhibition of gas exchange at low RZTs has been reported for several species, including conifers (DeLucia and Smith 1987, Day et al. 1989, 1990, 1991, Ryyppö et al. 1998). Cold soil prevents efficient uptake of water by roots by affecting the physical properties of water, by preventing biochemical changes in root cell membranes and by inhibiting root growth (Kramer 1983, Häussling et al. 1988). According to Häussling et al. (1988), a high rate of water uptake is confined to growing

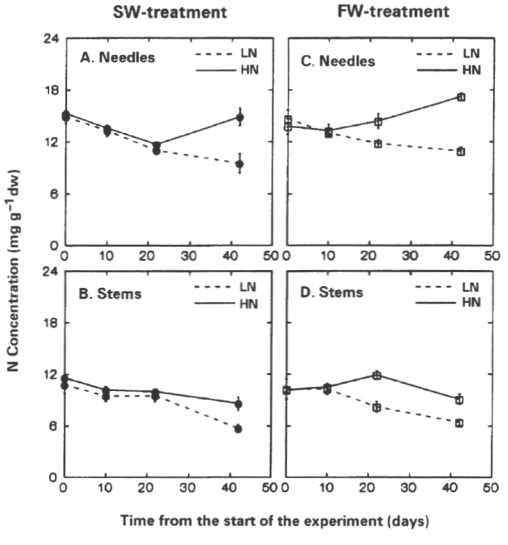

Figure 4. Nitrogen concentrations of needles and stems of Scots pine (*Pinus sylvestris*) seedlings during the 6-week experiment. The seedlings were exposed to slow (SW-treatment; A, B) or fast (FW-treatment; C, D) warming of the root zone and were grown at high (3 mM N; HN) or low (0.5 mM N; LN) nutrient availability. In the SW-treatment, RZT was 4, 5, 9 and 18 °C at Days 0, 10, 22 and 42, respectively. In the FW-treatment, RZT was 4, 12, 18 and 18 °C at Days 0, 10, 22 and 42, respectively. Data are means (± SE) of three replicates.

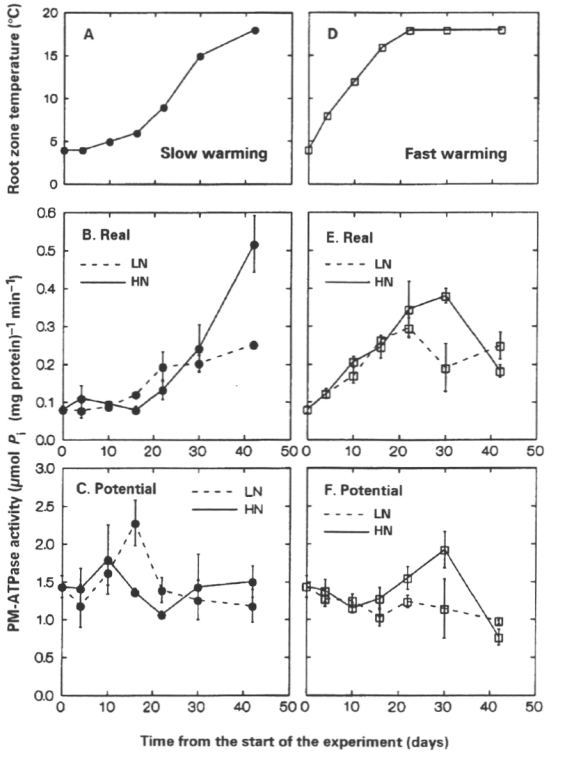

Figure 5. Real (B, E) and potential (C, F) PM-ATPase activity in Scots pine (*Pinus sylvestris*) roots during the 6-week experiment. The roots were exposed to slow (A) or fast (D) warming of the root zone and grown at high (3 mM N; HN) or low (0.5 mM N; LN) nutrient availability. Data are means (± SE) of three replicates.

root tips, and thus factors that inhibit root growth will also inhibit water uptake. Decreased water uptake limits net photosynthesis because shoot water deficit causes stomatal closure (Lopushinsky and Kaufmann 1984). In the present study, net photosynthesis, transpiration, and stomatal conductance were lower in seedlings in the LN-treatment than in the HN-treatment (Table 3). Similarly, nitrogen concentrations of needles and stem were lower in seedlings in the LN-treatment than in the HN-treatment (Figure 4). A decrease in gas exchange may be the result of decreased amount and activity of RuBP carboxylase and reduced concentrations of soluble protein and chlorophyll. All of these parameters are low in Scots pine seedlings grown under conditions of nutrient deficiency (Gezelius 1986). Nutrient availability had a significant effect on gas exchange in the SW-treatment on Day 22. At that time, RZT was only 9 °C and seedling growth had not responded to the HN-treatment; however, foliar nitrogen concentration was slightly higher in the HN-treatment than in the LN-treatment. The timing of these responses imply that nutrient uptake by seedlings in the HN-treatment began at low RZTs, leading to higher foliar nitrogen concentration and photosynthetic capacity in the HN-treatment compared with seedlings in the LN-treatment.

Data from several studies suggest that roots can acclimate to low RZTs by increasing their capacity for nutrient uptake, translocation and xylem exudation (Clarkson 1976, Siddiqi et al. 1984, Bigot and Boucaud 1996, Engels and Marschner 1996). For example, the capacity of roots to take up macronutrients like K (Siddiqi et al. 1984, White et al. 1987), N (Deane-Drummond and Glass 1983, Engels et al. 1992, Bigot and Boucaud 1996) and Ca (Engels et al. 1992) increases during exposure to low RZT. If ion uptake increases at low RZTs as a consequence of acclimation, PM-ATPase activity may also increase because of its role in active ion uptake. However, we did not observe an increase in potential PM-ATPase activity, indicating that the capacity of PM-ATPase does not change significantly as a consequence of long-term exposure to low RZT (Figure 5C). In contrast, real PM-ATPase activity increased with increasing RZT (Figures 5B and 5E). At low RZTs, real PM-ATPase depended on temperature and was independent of the external supply of nutrients and the nutrient demand of the shoot. At high RZTs, nutrient availability affected real PM-ATPase activity, which was almost doubled in the SW + HN treatment at an RZT of 18 °C (Figure 5B). The increase in real PM-ATPase activity, which has multiple functions in growing root cells, was correlated with an increase in root growth (Table 1, Figures 2C and 3B).

In Scots pine seedlings, low RZTs suppressed growth of roots and shoots, root PM-ATPase activity and gas exchange of the shoot. Gas exchange increased with increasing RZT, especially when nutrient availability was high, indicating the importance of seedling nutritional status in recovery after long-term exposure to suboptimal RZTs. We conclude that long-term inhibition of root growth by low RZT may be overcome and compensated for when RZT and nutrient availability become favorable for growth.

Acknowledgments

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III

Seasonal root growth of Scots pine seedlings in relation to shoot phenology, carbohydrate status, and nutrient supply

S. Iivonen, R. Rikala, and E. Vapaavuori

Abstract: To ascertain whether the growth rhythm of roots differs from that of the shoot, the seasonal pattern of dry mass allocation was determined in 1-year-old Scots pine (*Pinus sylvestris* L.) seedlings. Gas exchange, chlorophyll fluorescence, and the dynamics of starch and soluble sugars were examined to understand the role of stored carbon and that of current photosynthates in meeting the sink demand of plant organs. In this growth-chamber experiment, hydroponic cultures supplied the seedlings with low (0.25 mM N) or high (2.5 mM N) nutrient level. The climatic conditions in the chamber simulated the weather conditions from May to mid-October in southern Finland. Root growth was most intense at the end of the growing season, at which time shoot growth slowed down. Nutrient level did not affect the growth rhythm of the roots, but the total production of root biomass was favoured by high level of nutrients. The response of root growth to root zone temperature (RZT) was not the same over the growing season, indicating that the sensitivity of root growth to RZT depends on the growth phase of the seedling. The growth rhythm of the roots is probably regulated by several internal and external factors and their interactions, including RZT and availability of photosynthates.

Résumé : Les auteurs ont déterminé le patron saisonnier d'allocation de masse sèche chez des semis de pin sylvestre (*Pinus sylvestris* L.) âgés d'un an dans le but de vérifier si le rythme de croissance des racines diffère de celui des pousses. Les échanges gazeux, la fluorescence de la chlorophylle et la dynamique de l'amidon et des sucres solubles ont été examinés afin de comprendre le rôle des réserves de carbone et des produits courants de la photosynthèse face au puits que représente la demande par les organes de la plante. Dans cette expérience en chambre de croissance, les semis en culture hydroponique ont été exposés à des niveaux faible (0,25 mM N) ou élevé (2,5 mM N) de nutriments. Les conditions climatiques dans les chambres de croissance correspondaient aux conditions climatiques du mois de mai à la mi-octobre dans le sud de la Finlande. La croissance des racines était plus intense à la fin de la saison de croissance au moment où la croissance des pousses ralentissait. Le niveau de nutriments n'a pas affecté le rythme de croissance des racines mais la production totale de biomasse racinaire était favorisée par un niveau élevé de nutriments. La réponse de la croissance racinaire à la température dans la rhizosphère n'était pas la même pendant toute la saison de croissance. Cela indique que la sensibilité de la croissance racinaire à la température dans la rhizosphère dépend de la phase de croissance dans laquelle se trouve le semis. Le rythme de croissance des racines est probablement régulé par plusieurs facteurs internes et externes et leurs interactions, incluant la température dans la rhizosphère et la disponibilité des produits de la photosynthèse.

[Traduit par la Rédaction]

Introduction

In boreal regions, conifer seedlings are usually outplanted in early spring. Increasing use of planting machines and improved possibilities to control seedling growth in nurseries emphasizes the need to enlarge planting windows. Therefore, better knowledge of the seasonal pattern of root growth in relation to growth of other organs might serve in finding the optimal periods when root growth is favoured and would lead to rapid establishment of the seedling on a site. In northern conifers, the seasonal changes in growth of the aboveground organs are well-studied (Lanner 1967; Garret and Zahner 1973; Raulo and Leikola 1974; Junttila and Heide 1981; Dougherty et al. 1994; Gower et al. 1994), while less information is available about the root growth. Opinions about root growth rhythm during the growing season differ considerably, which is not surprising considering the wide range of sampling techniques, environmental conditions, and tree species used in different studies. According to several authors, there is no characteristic seasonal pattern of root growth (Persson 1978, 1980*a*; Ahlström et al. 1988; Makkonen and Helmisaari 1998), and fluctuations in root growth are caused by changes in environmental conditions (Persson 1978). On the other hand, it has been shown that the seasonal pattern of root growth differs from that of the shoot and that the variation in root growth during the growing season is closely linked to phenology and growth of the shoot (Krueger and Trappe 1967; Lyr and Hoffmann 1967; Drew and Ledig 1980; Sword et al. 1996). Root growth can continue even after shoot dor-

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mancy begins to develop; therefore, it has been suggested that cessation of root growth is determined mainly by decreasing root zone temperatures (RZT) at the end of the growing season (Lyr and Hoffmann 1967).

Plant growth and the distribution of biomass throughout the plant are dependent on allocation of assimilated carbon among various sinks and on the sink strength of the organs. Whether newly assimilated carbon or stored carbon is utilized in growth processes depends on the effectiveness of photosynthesis and on the carbon cost in growth and maintenance of metabolic functions (Luxmoore et al. 1995). Nutrient availability strongly affects growth of the needles (Rikala and Huurinainen 1990) and roots (Coutts and Philipson 1977); thus, the sink strength of the organs and the utilization of carbohydrates for growth and respiration may vary, depending on nutrient availability.

In this study, our objective was to determine the seasonal pattern of dry mass allocation among the organs of Scots pine (*Pinus sylvestris* L.) seedlings subjected to low or high nutrient level. The experiment was performed in a growth chamber where the conditions of "average growing season" could be simulated. Hydroponic culture was used and served as a model system enabling maintenance of constant supply of nutrients and water, as well as maintainance of the desired RZTs. Special emphasis was placed on questions of whether root growth is related to aboveground phenology and whether competition between various sinks results in a different seasonal growth rhythm in the root system compared with that of the shoot. Gas exchange of the seedlings, chlorophyll fluorescence of the needles, and dynamics of starch and soluble sugars were examined to ascertain the roles of stored carbon reserves and of current photosynthates in meeting the sink demand of the organs.

Materials and methods

Plant material

The experiment was conducted with 1-year-old Scots pine seedlings grown in peat-filled Ecopots (PS-508, Lännen Tehtaat Co., Finland; growing density 620 seedlings/m^2) in the nursery of Suonenjoki Research Station (62°40′N, 27°00′E). The seed material originated from a seed orchard (62°05′N, 26°10′E) established with material obtained from different sites in Finland between 61°70′N and 64°20′N. In mid-October 1996 the seedlings were transferred to cold storage and were kept there at –3°C over winter. In the beginning of February 1997, the seedlings were thawed in darkness at 4°C for 2 weeks. Thereafter, the peat surrounding the root system of the seedlings was removed by gently washing the roots with cold tap water. The seedling population used in the study was standardized by rejecting the shortest and longest seedlings, and in the experiment the initial shoot length of the seedlings was 14.8 ± 1.8 cm (mean ± SD).

Experimental design

Immediately after root washing, a total of 1152 seedlings were placed into 12 plastic containers (volume 40 dm^3, 96 seedlings per container) in a growth chamber (Weiss, type 10 Sp/5 DU-Pi, Lindenstruth, Germany). The containers were filled with aerated nutrient solution containing a low level of nutrients (0.25 mM N and other essential nutrients in proportion to nitrogen) (Ingestad and Lund 1986). Air and root zone temperatures were adjusted to 4°C and the seedlings were kept in continuous dim light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) during the first 3 days. Thereafter, the containers were grouped into three blocks, each having four containers. Each block was then divided into two nutrient treatments: low nutrient level (0.25 mM N; LN treatment) and high nutrient level (2.5 mM N; HN treatment), two containers for each. In the nutrient solutions (Ingestad 1979; Ingestad and Lund 1986), nitrate and ammonium ions made up 58 and 42% of the total nitrogen, respectively. The concentrations of all other essential nutrients were supplied in proportion to the amount of nitrogen. The mass proportions of macronutrients in the nutrient solutions were 100 N : 60 K : 18 P : 6 Ca : 6 Mg : 9 S. The pH of the nutrient solutions was adjusted to 4.5–5.5 by adding 1 M NaOH or 1 M HCl. To maintain target nutrient concentrations, electrical conductivity (40–50 and 240–270 µS in LN and HN treatment, respectively) and pH of nutrient solutions were measured twice a week and according to these measurements, when necessary, nutrient solutions were added. Also, the concentrations of NH_4-N and NO_3-N of the nutrient solutions were checked by liquid chromatography (Flow Injection Analyzer, Tecator, Höganäs, Sweden) several times during the experiment and at monthly intervals the total nutrient solutions were changed. During the experiment, the nutrient solutions were continuously bubbled with compressed air (Iivonen et al. 1999) to ensure good supply of oxygen for roots.

The daily and seasonal fluctuation in air and root zone temperature, relative air humidity, and light intensity were the same in all treatments. Air and root zone temperature and photoperiod were simulated, with slight modifications, to follow the average weather and light conditions in Jokioinen (60°49′N, 23°30′E), based on meteorological data measured during 1961–1990 (Finnish Meteorological Institute 1991) (Fig. 1). The simulated growing season started immediately after a 3-day adjustment period. In the simulated conditions, the length of a month was 26 days; and the total temperature sum developed during the simulated growing season was 1044 degree-days (DD; threshold of 5°C) (Fig. 1), which was the lower limit in the temperature sum zone of 1000–1300 DD in southern Finland between 64 and 60°N (Statistical Yearbook of Forestry 1998). Relative air humidity in the growth chamber was 60–80% in May–June, 60–90% in July–August, and 70–90% in September–October, being lowest in the middle of the day and highest in the middle of the night. To control the root zone temperature, a water-filled plastic coil was placed into the bottom of each container. The coils were connected to cryostats (MGW, Lauda, Germany) that circulated water in the coil, thus maintaining the desired temperature in the nutrient solutions. The temperature of the nutrient solutions was measured daily with a digital thermometer (Sensotherm 100, Nürnberg, Germany). Air temperature and relative humidity in the chamber were recorded continuously by a thermohygrograph. Maximum daily photon flux density at the top of the seedlings was 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR) (LI-185B, LI-COR, Inc., Lincoln, Nebr.).

Growth and nitrogen analyses

Before the growing season (day 0 samples), the dry mass was determined by weighing 96 randomly sampled seedlings. During the experiment, the dry mass was determined nine times by weighing at each time eight seedlings per container, which means a total of 48 seedlings per nutrient treatment. Current- and previous-year roots, needles, and stems of the eight seedlings were separated and weighed, and thereafter, the organs were pooled and stored at –80°C. Current roots were separated when the length of the new roots was >1 cm. Dry mass of roots was determined from a subsample of frozen, homogenized roots that was then dried at 60°C for 48 h. After drying, the nitrogen concentration of the needle and stem samples was measured with a CHN-600 analyzer (Leco Co., St. Joseph, Mich.) and that of the root samples with a CHN-900 analyzer (Leco Co., St. Joseph, Mich.). For the results, data of nitrogen concentration were recalculated as N concentration of shoot and roots. The length of the stem was measured repeatedly from five seed-

lings per container (altogether 30 seedlings per treatment) at the beginning of the experiment and nine times after that, and the length of a single current needle was measured repeatedly from five seedlings per container 12 times during the experiment.

Gas exchange and chlorophyll fluorescence measurements

The gas exchange of the whole shoot was determined (18 seedlings/treatment) in repeated measurements with a closed-system infrared gas analyser (LI-COR 6200, LI-COR, Lincoln, Nebr.) five times during the experiment. During the measurements, the root system of the seedlings was maintained at same temperature and kept in the same nutrient solution as in the growth chamber. The shoot temperature was 22°C and saturating light 700 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR (cool beam halogen lamps FTY GX5.3, Sylvania, Belgium). Gas exchange was measured for 1 min after a constant relative humidity of 40–70% and CO_2 concentration of 360 $\mu L \cdot L^{-1}$ were reached. During each measurement period, relative humidity was the same in the two nutrient treatments, with the exception of the August measurement when RH was 19% higher in the HN treatment than in the LN treatment. Gas-exchange rate was calculated on the basis of the silhouette area of the shoot, which was measured after each gas-exchange measurement using a video camera connected to a graphic monitor.

The fluorescence induction of needles at room temperature were measured with a MINI-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany). During the experiment, measurements were made on current- and previous-year needles of eight seedlings per container (altogether 48 seedlings per treatment) six and nine times, respectively. Each sample consisted of about 10 needles from each seedling. The needles were dark adapted at room temperature for 20 min before fluorescence was measured using a 1-s saturating pulse of 9000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The ratio of variable (F_v) to maximal (F_m) chlorophyll fluorescence was used as a quantitative measure of the photochemical efficiency of the photosystem II (PS II).

Carbohydrate analyses

Soluble sugars and starch were analyzed in pooled samples of current- and previous-year needles, stems, and roots of eight seedlings seven times during the experiment. A total of six pooled samples per treatment were dried at 60°C for 48 h and ground to a powder. The samples were extracted and analyzed as described by Hansen and Møller (1975). Soluble sugars were extracted from a ground sample (30–40 mg dry mass) with a total volume of 15 mL of 80% aqueous ethanol. First, 3 mL of ethanol was added to the sample, which was then heated for 5 min at 60°C and centrifuged for 4 min at 1880 × *g*. The supernatant was collected and the extraction procedure was repeated four times. Total soluble sugars in the pooled supernatant were determined colorimetrically at 630 nm with anthrone using D-glucose (alpha-D-glucose, anhydrous, analytical grade, Serva) as a standard. Starch was extracted from the residue with 20 mL of 30% perchloric acid. The residue was first shaken for 20 h at room temperature with 10 mL of 30% perchloric acid. After centrifugation for 4 min at 1880 × *g*, the supernatant was collected. The extraction was repeated twice with 5 mL of 30% perchloric acid. From the pooled supernatant, starch was determined colorimetrically at 625 nm with anthrone and using starch (Starch soluble, pro analysis, Merck) in 30% perchloric acid as a standard.

Statistical analyses

The mean values for dry mass of the organs, the proportion of dry mass increment allocated to the roots, gas exchange parameters, F_v/F_m ratio, and needle and stem length of the sampled seedlings in each container were used as independent observations in the statistical analysis ($n = 6$). For statistical analysis of soluble sugars and starch, each measurement was used as an independent observation. For each variable, the homogeneity of variance among treatments was tested using Bartlett's test (Sokal and Rohlf 1981). When there was heteroscedasticity, the data were transformed to logarithms using the equation, $X' = \log(X + 1)$. To test whether growth of the roots is periodic, the proportion of dry mass increment allocated to the roots was used as a test parameter. Before statistical analysis, the proportions were transformed using angular transformation of the square roots of the values. Three-way analysis of variance was used to test the effects of nutrient level, block factor, and sampling day on shoot and root dry mass, gas exchange of the seedlings, chlorophyll fluorescence of the needles, needle length, and concentration of soluble sugars and starch. Untransformed mean values are shown in the figures. When the effects of nutrient level and sampling day on stem length were tested, three-way analysis of covariance was used with nutrient level, sampling day, and block as factors and initial stem length as covariate. Because gas exchange and needle and stem length were measured repeatedly from the same seedlings, in the statistical analyses the values for these parameters were regarded as repeated measures. The analyses were conducted using SYSTAT for Windows, version 5.0 (SYSTAT, Inc. 1992).

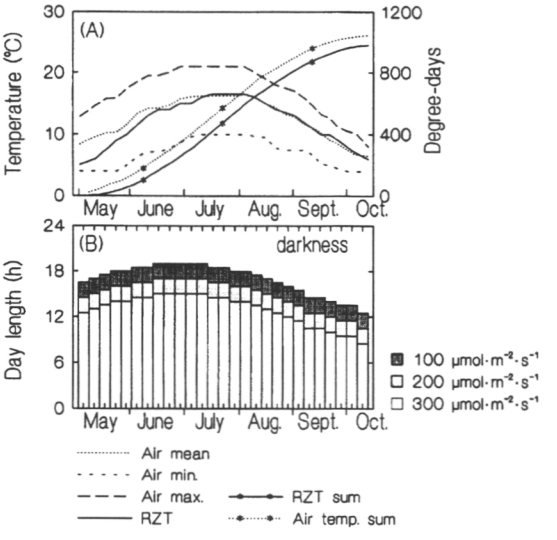


Fig. 1. Air and root zone temperature (RZT) sum in degree-days (days above a threshold of 5°C); minimum, maximum, and mean daily air; and mean RZT (A) and length of the photoperiod (B) in the growth chamber during the experiment. Seedlings were kept in dim light for 2 h before (1 h at 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ + 1 h at 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and after (1 h at 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ + 1 h at 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) a period of full light (300 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Results

Nitrogen concentration of shoot and roots

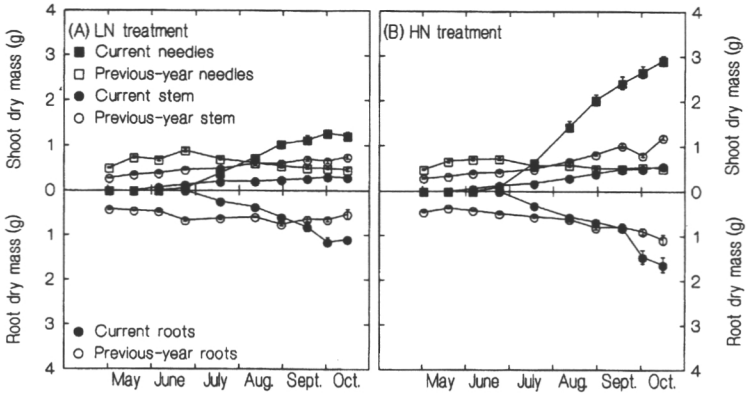
The initial nitrogen concentration [N] of shoot and roots was approximately 1.1 and 1.3%, respectively (Table 1). During the growing season, the effects of the two nutrient treatments were clear the HN treatment causing a significantly higher [N] in shoot and roots. In the LN treatment, [N] of shoot decreased during the growing season. In the HN treatment, [N] of shoot decreased until early June and, there-

Table 1. Nitrogen concentration (% of dry mass) of shoot and roots during the growing season.

	LN treatment		HN treatment	
Date	Shoot	Roots	Shoot	Roots
May 1	1.15±0.04	1.35±0.03	1.10±0.04	1.26±0.03
May 18	0.97±0.03	1.61±0.04	1.01±0.05	2.01±0.05
June 5	0.82±0.01	1.52±0.07	0.91±0.03	1.89±0.08
July 17	0.71±0.04	1.74±0.07	1.22±0.04	2.37±0.02
August 29	0.69±0.02	1.70±0.10	1.24±0.03	2.53±0.07
September 17	0.65±0.03	1.47±0.08	1.24±0.04	2.37±0.06
October 15	0.63±0.02	1.40±0.04	1.21±0.03	2.58±0.06

Note: Values are mean ± SE of six replicates. Scots pine seedlings were grown at low (0.25 mM N; LN treatment) or high (2.5 mM N; HN treatment) nutrient level.

Fig. 2. Mean cumulative dry mass of current and previous-year needles, stem, and roots of Scots pine seedlings during the simulated growing season. Seedlings were grown at low (A; LN treatment; 0.25 mM N) or high (B; HN treatment; 2.5 mM N) nutrient level. Values are mean ± SE of six replicates, each consisting of eight sample seedlings.



after, increased to value of 1.2%, which was maintained until the end of the growing season.

Dry mass allocation to shoot and phenology of the shoot

The dry mass of current needles, previous-year needles, current stem, and previous-year stem were all significantly affected both by sampling day ($p < 0.001$) and by nutrient level ($p < 0.001$, $p = 0.011$, $p < 0.001$, and $p < 0.001$, respectively; Figs. 2A and 2B). There were also significant interactions among factors for current and previous-year stem ($p < 0.001$) and current and previous-year needles ($p < 0.001$ and $p = 0.001$; Figs. 2A and 2B). Stem length was significantly affected by sampling day ($p < 0.001$) but not by nutrient level, whereas the length of the current needles was significantly affected by both sampling day ($p < 0.001$) and nutrient level ($p < 0.001$), and their interaction ($p < 0.001$) (Fig. 5). During the growing season most of the total cumulative dry mass increment was allocated to the shoot (Figs. 3A and 3B), and only at the end of the growing season did allocation to the roots exceed that to the shoot (Figs. 4A and 4B). In early May, the total dry mass of the previous-year needles and stem increased (Figs. 2A and 2B) but this increase was mainly due to accumulation of nonstructural carbohydrates while increment of structural dry mass was slow until mid-June (Figs. 3A and 3B). Elongation of the stem started in mid-May and continued until mid-July (Fig. 5A). Current needles started to elongate in mid-June, and their growth ceased at the end of August in the LN treatment but continued until the end of growing season in the HN treatment (Fig. 5B).

Dry mass allocation to roots

The dry mass of current and previous-year roots was significantly affected by sampling day ($p < 0.001$) and nutrient level ($p < 0.001$ and $p = 0.005$, respectively) and by their interaction ($p = 0.018$ and $p < 0.001$, respectively; Figs. 2A and 2B). Growth of the roots accelerated in June (Figs. 3A and 3B), and the current roots flushed rapidly at the end of June after a period of rapid shoot elongation (Figs. 2A, 2B, and 5A). The proportion of dry mass increment allocated to the roots was significantly affected by sampling day ($p < 0.001$) and decreased at the time of intense needle elongation, which was from the end of June to mid-August in the LN treatment and from mid-July to mid-September in the HN treatment (Figs. 4A, 4B, and 5B). The increment in root dry mass was highest when the increment in shoot dry mass was retarded (Figs. 3A, 3B, 4A, and 4B). In LN treatment, growth of the roots peaked at the end of August and at the end of September (Fig. 4A), while in the HN treatment there was only one peak at the end of September (Fig. 4B). However, the differences between nutrient treatments were not

Fig. 3. Cumulative total and structural dry mass increment of shoot and roots of Scots pine seedlings during the simulated growing season. Structural dry mass = total dry mass (g) – content of soluble sugars and starch (g). Scots pine seedlings were grown at low (A; LN treatment; 0.25 mM N) or high (B; HN treatment; 2.5 mM N) nutrient level. Values are mean ± SE of six replicates, each consisting of eight sample seedlings.

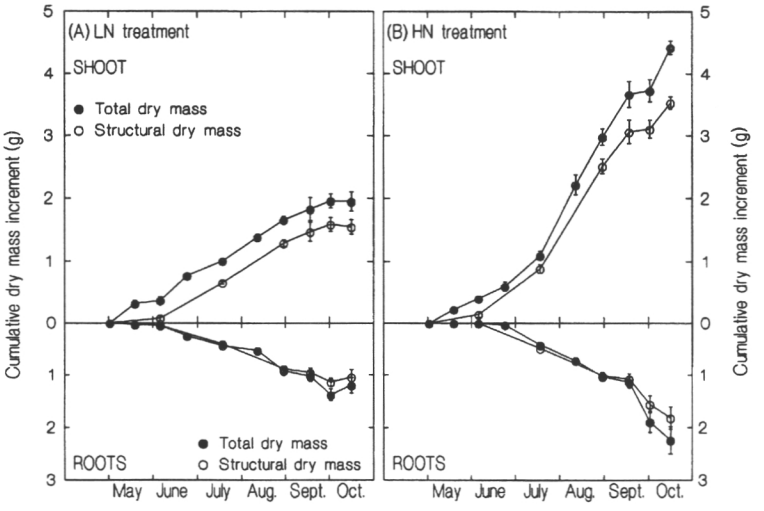
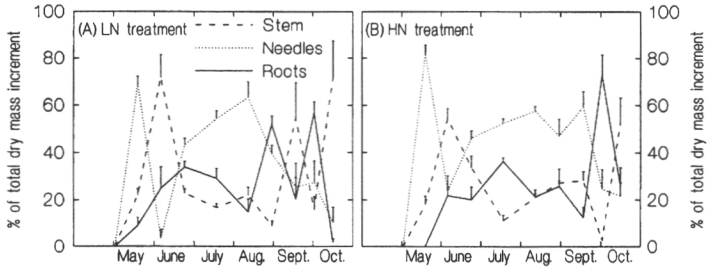


Fig. 4. Distribution of dry mass increment between needles, stem, and roots during the simulated growing season, expressed as a percentage of total dry mass increment. Scots pine seedlings were grown at low (A; LN treatment; 0.25 mM N) or high (B; HN treatment; 2.5 mM N) nutrient level. Values are mean + SE of six replicates, each consisting of eight sample seedlings.



statistically significant (Figs. 4A and 4B). Interestingly, no accumulation of nonstructural carbohydrates occurred in the root system until the end of September when in both nutrient treatments the content of soluble sugars and starch increased markedly (Figs. 3A, 3B, 7E, and 7F).

Gas exchange and chlorophyll fluorescence

The effect of nutrient level and sampling day, and their interaction, on net photosynthesis and maximum photochemical efficiency of PSII (F_v/F_m) was statistically significant ($p < 0.001$). In both nutrient treatments, net photosynthesis was slow until the end of June (Fig. 6A). Thereafter, in the HN treatment, net photosynthesis increased rapidly and was high until the beginning of October. In the LN treatment, net photosynthesis also increased but was about half of that in the HN treatment. Intercellular CO_2 concentration started to increase at the end of June and continued to increase until the end of the experiment, being significantly affected by both the sampling day ($p < 0.001$) and nutrient level ($p < 0.001$; Fig. 6D). Stomatal conductance (Fig. 6B) and transpiration (Fig. 6C) were low until the end of June and, thereafter, increased significantly ($p < 0.001$).

Already 2 weeks after the start of the experiment the effect of nutrients was seen as lower F_v/F_m values in the previous year needles of the LN treatment (Fig. 6E), and a similar difference between nutrient treatments was also seen in the F_v/F_m ratio of the current-year needles (Fig. 6F). There was also a strong seasonal variation in the maximum photochemical efficiency of PSII: in spring F_v/F_m increased rapidly with highest photochemical efficiency at the beginning of August and, thereafter, decreased significantly, especially in the LN treatment.

Soluble sugar and starch contents

In current needles the content of soluble sugars was affected by sampling day and nutrient level ($p < 0.001$). It increased until the end of September and declined rapidly in October in both nutrient treatments being all the time higher in HN treatment (Fig. 7A). In the current and previous-year stem the content of soluble sugars was affected by sampling

Fig. 5. Length of stem (A) and current needles (B) of Scots pine seedlings during the simulated growing season. Seedlings were grown at low (LN; 0.25 mM N) or high (HN; 2.5 mM N) nutrient level. Values are mean ± SE of six replicates, each consisting of five sample seedlings or needles. HN treatment, solid symbols; LN treatment, open symbols.

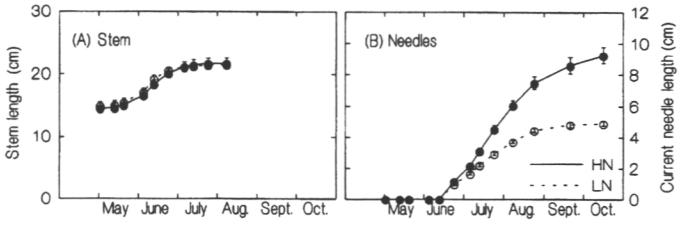
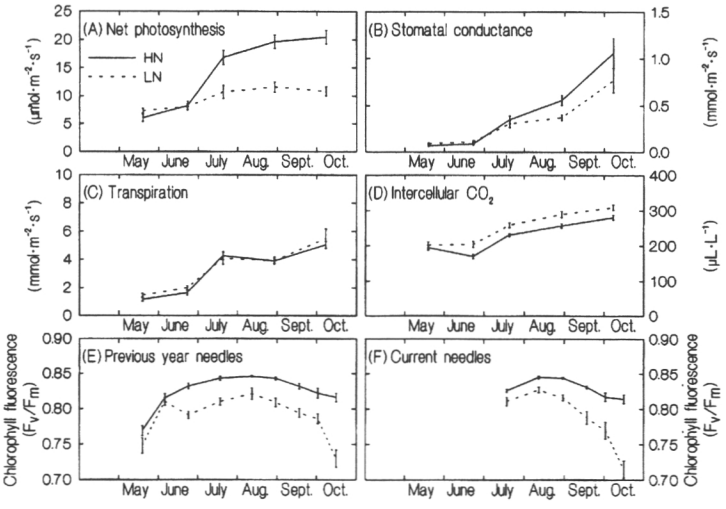


Fig. 6. Net photosynthesis (A), stomatal conductance (B), transpiration (C), intercellular CO_2 (D), and chlorophyll fluorescence of the previous-year (E) and current (F) needles of Scots pine seedlings during the simulated growing season. Seedlings were grown at low (LN; 0.25 mM N) or high (HN; 2.5 mM N) nutrient level. Values are mean ± SE of six replicates, each consisting of three sample seedlings.



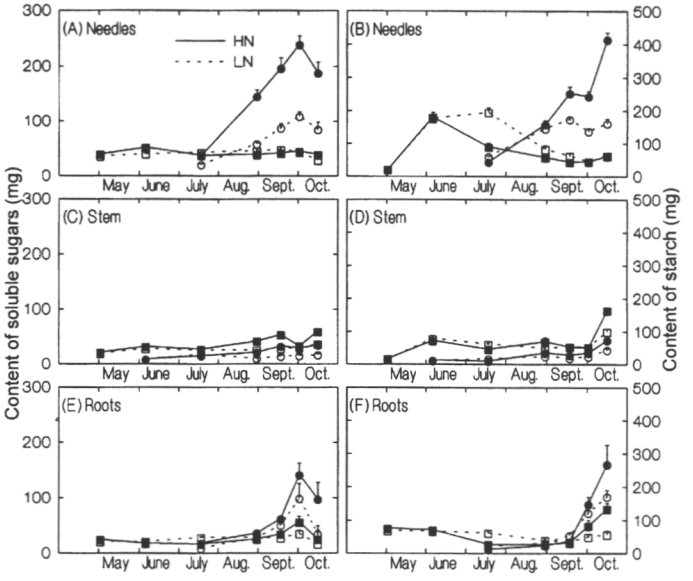
time, nutrient level and their interaction ($p < 0.001$). Accumulation of soluble sugars was particularly marked in HN treatment from mid-July to the end of the growing season (Fig. 7C). The content of soluble sugars in current roots was affected by sampling day ($p < 0.001$) and nutrient level ($p = 0.004$), but in the previous year roots it was affected only by sampling day ($p < 0.001$). Sugar content of the roots was low until the end of August (Fig. 7E), when it started to increase peaking at the end of September and decreasing again in October.

The starch content of current and previous-year needles was affected by sampling day ($p < 0.001$), nutrient level ($p < 0.001$), and their interaction ($p = 0.004$ and $p < 0.001$, respectively). The starch content of previous-year needles and stem increased 8.5- and 4.3-fold in May, respectively, (Figs. 7B and 7D). When shoot growth started, starch content of the needles in the HN treatment declined, while in the LN treatment the starch content of needles was high until mid-July and even afterwards remained higher than in the HN treatment. The starch content of current needles increased from mid-July onwards and was 2.7- and 9.4-fold higher at the end of the growing season in the LN and HN treatments, respectively (Fig. 7B). Starch contents of the current and previous-year roots were both affected by sampling day ($p < 0.001$) and also by nutrient level ($p < 0.001$) in the previous-year roots. The starch content of roots was high at the beginning of the experiment (Fig. 7F) but declined in both nutrient treatments the decline being more rapid in the HN treatment. However, at the end of the growing season there was a marked accumulation of starch in the root system, and then starch content was significantly higher in HN than in LN treatment.

Discussion

In natural growing conditions the examination of seasonal root growth in trees is laborious, and because of different methods, studies have given contrasting results. In the present study we used hydroponic culture technique as a model system that enabled continuous examination of the roots with simultaneous measurements of the shoot growth and physiological performance. The experimental technique also allowed us to simulate a full growing season using the long-term records of air and soil temperature, relative humidity,

Fig. 7. Soluble sugar contents of current (circles) and previous-year (squares) needles (A), stem (C), and roots (E) and starch contents of current (circles) and previous-year (squares) needles (B), stem (D), and roots (F) of Scots pine seedlings during the simulated growing season. Seedlings were grown at low (LN; 0.25 mM N; open symbols) or high (HN; 2.5 mM N; solid symbols) nutrient level. Values are mean + SE of six replicates.

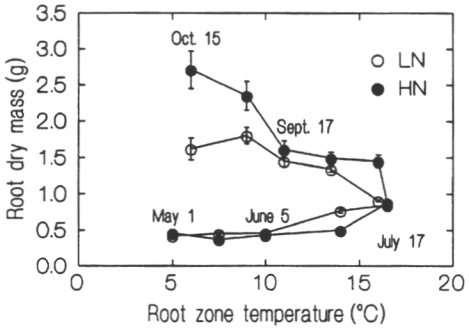


and photoperiod. Our growing season simulation shows that, in Scots pine seedlings, growth of stem, needles, and roots occur in phases, each having their own "window of dry mass accumulation". The episodic growth of shoot and roots has been found also in the seedlings of other northern pines, *Pinus contorta* Dougl. ex Loud. (Cannel and Willett 1976) and *Pinus resinosa* Ait. (Drew 1982). According to previous studies with 15- to 20-year-old Scots pine trees it was concluded that no part of the growing season is especially favourable for root growth, and root growth takes place whenever soil moisture and temperature conditions permit (Persson 1978, 1980*a*). Our results with Scots pine seedlings do not support this suggestion; in hydroponic culture where the drought effect was excluded and root zone temperature was controlled, root growth demonstrated a clear periodic behaviour. It is difficult to assess whether the conflicting results are due to different ontogenetic phase of the trees, since at present there are no results of simultaneous measurements with seedlings and bigger trees.

The dry mass growth of a plant organ can be divided to growth of structural biomass and increase or decrease of nonstructural carbohydrate reserves. In Scots pine seedlings the growth rate was low in May, and the production of photosynthates was higher than the demand. This led to accumulation of starch in the previous-year needles and stems, causing an increase in the dry mass of the previous year needles. In contrast to the aboveground parts, starch content in the roots was already high after winter storage, and there was no net nonstructural carbohydrate accumulation in May. In conflict with our results, however, several authors have reported of starch accumulation in the roots of well-established conifers before bud break (Krueger and Trappe 1967; Ericsson and Persson 1980; Deans and Ford 1986). In the present study, seedlings were kept in winter storage (–3°C) for 3.5 months before the start of the experiment, and thus, only small amounts of carbohydrate reserves might have been lost in maintenance respiration during storage period if compared with normal winter conditions. Because starch content of the roots was high already at the beginning of the experiment, the root system did not serve as a sink, and net translocation of solutes could not be observed in May.

In June and early July, when shoot elongation was rapid and elongation of the current needles had initiated, the starch content of previous-year needles and stem declined with the exception of previous-year needles in the LN treatment. Previous studies also show that shoot growth is facilitated by current photosynthates and that reserves from the previous autumn play only a minor role in shoot elongation (Hansen and Beck 1994; Lippu 1998). In the HN treatment, increased nitrogen concentration of the shoot led to increased photosynthesis and content of soluble sugars; therefore, growth was accelerated. In contrast, in the LN treatment, nitrogen deficiency decreased the photochemical efficiency of PSII and net photosynthesis leading to reduced growth and starch accumulation. According to Ericsson (1979), fertilization increases the sink activity of the tree, causing either decreased rate of accumulation or increased utilization of starch reserves. Adams et al. (1986) also demonstrated that loblolly pine with high foliar nitrogen concentration uses starch reserves earlier in the growing season compared with pines with low nitrogen concentration in the foliage. In the present study, starch reserves in the previous-year needles were probably consumed during elongation of the current needles, which occurred more rapidly in the HN treatment than in the

Fig. 8. Root dry mass versus root zone temperature of Scots pine seedlings during the simulated growing season. Seedlings were grown at low (LN; 0.25 mM N) or high (HN; 2.5 mM N) nutrient level. Values are mean ± SE of six replicates, each consisting of eight sample seedlings.



LN treatment. Therefore, the more rapid hydrolysis of starch reserves reflects enhanced metabolic activity and growth when the nutrient supply is ample.

The elongation of current needles started in mid-June, and a month later, net photosynthesis of the whole shoot had increased considerably; the differences between nutrient treatments were also apparent. In the early stages of development, net photosynthesis of current needles is low, and they are net importers of current photosynthates (Troeng and Linder 1982). Only when current needles have reached half of their final length is the carbohydrate production of the current needles high enough to meet the demand of the other organs (Ericsson 1978). In the present study the whole shoot was enclosed in the chamber during the gas-exchange measurements, and therefore, the contribution of different needle groups cannot be separated. Our measurements show that current needles reached half of their final length at the end of July, when also their dry mass reached the same value as in the previous-year needles. According to previous studies (Ericsson 1979; Gezelius and Hallen 1980, Vapaavuori et al. 1995), we reason that, at the end of July and onwards, most of the measured net photosynthesis was due to current needles. If the carbohydrate production of the current needles is not high enough to meet the demand of other organs until the end of July (Ericsson 1978), then this means that the photosynthates produced by the previous-year foliage, the starch reserves, or both are important for root growth during the first half of the growing season. According to an earlier suggestion (Vapaavuori et al. 1992), carbohydrate reserves can be used for formation of root tips, but the current photosynthates are needed for root elongation. In the present study, formation of new root tips in early June coincided with a decrease in the starch content of the roots, which indicates that carbohydrate reserves can be used for root tip formation. Increased dry mass allocation to the current roots was seen at the end of June, at the same time as elongation of the stem was almost completed. Also a previous study with 1-year-old Scots pine seedlings shows that root growth capacity of 1-year-old Scots pine seedlings is depressed during the time of intensive shoot elongation (Mattsson 1986). Therefore, we suggest that current photosynthates became available for root growth only after the sink activity of the elongating stem decreased.

In midsummer, during the time of formation of new needles, only 15–36% of total dry mass increment was allocated to roots. At that time, root growth was composed of structural dry mass with no net accumulation of nonstructural carbohydrates to roots. This confirms the suggestion that the root growth is slowed down when new needles are formed, because growing shoots are strong consumers of assimilates in Scots pine seedlings (Lyr and Hoffmann 1967). In both nutrient treatments, growth of the current needles continued in August, when net photosynthesis and the photochemical efficiency of PSII of the needles reached their maximum. Nutrient treatment had a marked effect on the chlorophyll fluorescence of current and previous-year needles, and apparently the lower net photosynthesis in LN treatment was partly due to the lower photochemical efficiency of PSII. The higher intercellular CO_2 concentration in the LN treatment compared with the HN treatment indicates that the rate of carboxylation also limited net photosynthesis in the LN treatment. Stomatal conductance and transpiration did not differ significantly between nutrient treatments, indicating that there was no stomatal limitation of photosynthesis in the LN treatment. At the end of August, elongation of the current needles ceased in the LN treatment and was delayed in the HN treatment. Thereafter, the dry mass increment of the shoot in the LN treatment was mainly due to diameter growth of the stem and starch accumulation and, in the HN treatment, also due to needle elongation. After shoot growth had ceased, the dry mass allocation to the root system was accelerated. Simultaneously with a shift in the allocation of below- and above-ground biomass, the content of soluble sugars increased significantly in the current needles and roots. The excess current photosynthates formed in the needles were eventually translocated to the roots. At this stage, translocation of carbohydrates to roots was greater than could be used in structural growth leading to accumulation of nonstructural carbohydrates. Since at the same time, substantial amounts of nonstructural carbohydrates also accumulated in aboveground organs, the proportion of structural dry mass increment allocated to root system was high, 49 and 73% of the total structural dry mass increment in LN and in HN treatment, respectively (data not shown).

The most intensive period of root growth was at the end of the growing season after the major part of shoot growth had taken place. By that time the RZT had already started to decrease but was higher than air temperature. At low RZTs in spring, root growth is low (Vapaavuori et al. 1992; Ryyppö et al. 1998; Iivonen et al. 1999), and this was also demonstrated in the present study. Our results from the present study show that the response of root growth to RZT does not stay the same throughout the whole growing season, and RZTs that inhibit or retard growth in spring can be favourable for root growth in the autumn (Fig. 8). This indicates that the sensitivity of root growth to RZT depends on the developmental stage of the seedling. Our results also show that, in both nutrient treatments, there was competition between shoots and roots for photosynthates, but nutrient availability did not significantly affect the pattern of dry mass allocation to roots. However, the total growth of root dry mass was greater in HN treatment than in LN treatment,

mainly due to later cessation of root growth in the HN treatment. Persson (1980*b*) has also reported that, in a Scots pine stand with near-optimum availability of nutrients and water, fine roots continue to grow for a longer time.

In conclusion, the results of this study, performed in the hydroponic culture, show that root growth is closely linked to shoot growth in Scots pine seedlings. The coincidence of retarded shoot growth and intense root growth indicates that root growth is controlled internally by availability of photosynthates during the time when decreasing RZT does not significantly restrict the metabolic processes in the roots. It is well known that hormones are important in internal coordination of growth and development of different organs (see Davies 1995; see Lambers et al. 1998), and therefore, a hormonal mechanism may have a central role in controlling growth allocation between shoot and roots. Thus, root growth is probably regulated by several internal and external factors and their interactions, including RZT and availability of photosynthates. The response of root growth to environmental factors is dependent on the growth phase of the seedling and, therefore, can differ at different times of the growing season. In the present study this was clearly demonstrated as the different growth response of roots to RZT in spring and in autumn.

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IV

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Seasonal variation in nitrogen net uptake and root plasma membrane H^+-ATPase activity of Scots pine seedlings as affected by nutrient availability

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Summary We examined changes in nitrogen (N) net uptake and activity and amount of plasma membrane H^+-ATPase (PM-ATPase) in roots of hydroponically cultured Scots pine (*Pinus sylvestris* L.) seedlings throughout a simulated second growing season. Seedlings were grown with low (0.25 mM N) or high (2.5 mM N) nutrient availability to determine whether root PM-ATPase is dependent on an external nutrient supply. Climatic conditions in the growth chamber simulated the mean growing season from May to mid-October in southern Finland. Root PM-ATPase activity varied considerably during the growing season and was higher in current-year roots than in previous-year roots. Total PM-ATPase activity of current-year roots was highest at the end of the growing season, whereas PM-ATPase activity per unit fresh mass of current-year roots and specific absorption rate of N were highest in mid-July and decreased at the end of the growing season. This indicates that the decrease in PM-ATPase activity per unit fresh mass of the roots at the end of the growing season was compensated by the increased size of the root system. Seasonal variation in PM-ATPase activity had no clear dependence on root zone temperature. The response of PM-ATPase to root zone temperature was dependent on the developmental stage of the seedling. High nutrient availability resulted in increased root PM-ATPase activity and an extended period of root growth in autumn.

Keywords: hydroponic culture, Pinus sylvestris, potential activity, real activity, root zone temperature, shoot.

Introduction

Nutrient uptake is influenced by environmental factors including nutrient supply (Jensén and Pettersson 1980), soil water (Oren and Sheriff 1995) and root zone temperature (RZT) (Bowen 1970, Engels and Marschner 1996*a*, Iivonen et al. 1999), and it is also regulated internally. Acclimation of uptake and translocation of nutrients to growth-related demand has been described for several macro- (Engels et al. 1992, Imsande and Touraine 1994, Engels and Marschner 1996*b*) and micronutrients (Engels and Marschner 1996*a*). In conifer seedlings, the demand for mobile nutrients for initial growth can also be satisfied by remobilization of nutrients from older parts of the plant (Millard and Proe 1993).

During growth and maturation, roots undergo extensive anatomical changes that greatly affect their permeability to water and solutes (Kramer 1983). Although water (Häussling et al. 1988) and ion uptake (Chung and Kramer 1975, Jensén and Pettersson 1980) occur primarily in root apical zones where the roots are newly formed and unsuberized, the importance of older suberized roots in water and nutrient uptake should not be underestimated (Chung and Kramer 1975, Van Rees and Comerford 1990, MacFall et al. 1991). During the growth process, the most active part of the root system is continuously renewed, and at the same time the activity of the older parts decreases. Because of the dynamic behavior of the root system, its nutrient uptake activity may vary considerably during the growing season.

Plasma membrane H^+-ATPase (PM-ATPase) plays a major role in nutrient uptake and loading because it pumps protons outside the plasma membrane, producing an electrogenic proton gradient across the membrane and thus providing energy to secondary transporters (Serrano 1990, Michelet and Boutry 1995). The expression of root PM-ATPase is regulated by root developmental stage and environmental conditions (Roldán et al. 1991). The activity of the enzyme is dependent on environmental conditions, including RZT (Ryyppö et al. 1998, Iivonen et al. 1999) and nutrient availability (Kuiper et al. 1991, Santi et al. 1995, Qiu and Su 1998), but may also be stimulated by the growth hormones auxin (Gabathuler and Cleland 1985, Santoni et al. 1991) and cytokinin (Kuiper et al. 1991).

Recently, we found that the most intensive period of dry mass allocation to roots in Scots pine (*Pinus sylvestris* L.) seedlings is at the end of the growing season after the period of intense shoot growth, at which time RZT is already decreasing (Iivonen et al. 2001). Because nutrient uptake must match the nutrient requirement of the seedling for growth, acclimatory changes must occur in the root system to meet the growth de-

mand for nutrients throughout the growing season.

The present study was undertaken to determine (1) the time of the growing season when roots are metabolically most active; (2) the relative roles of current- and previous-year roots in the metabolic activity of the root system; and (3) the relationship between metabolic activity of roots and RZT. We focused on the activity and amount of PM-ATPase in current- and previous-year roots as an indicator of metabolic activity, and on nitrogen (N) net uptake and growth of seedlings throughout the simulated growing season. In addition, the seedlings were grown with low or high nutrient availability to determine the importance of nutrient supply for growth and metabolic activity of the root system.

Material and methods

Plant material

The experiment was conducted with 1-year-old Scots pine (*Pinus sylvestris*) seedlings grown in peat-filled Ecopots (PS-508, Lännen Tehtaat, Iso-Vimma, Finland; growing density 620 seedlings m^{-2}) in the nursery at Suonenjoki Research Station (62°40′ N, 27°00′ E). The seed originated from a seed orchard (62°05′ N, 26°10′ E) established with material obtained from different sites in Finland between 61°70′ N and 64°20′ N. In mid-October 1996, the seedlings were transferred to cold storage and kept at –3 °C over winter. At the beginning of February 1997, the seedlings were thawed in darkness at 4 °C for 2 weeks. Thereafter, the peat surrounding the root systems of the seedlings was removed by gently washing the roots with cold tap water.

Experimental design

A total of 1152 seedlings were placed in 12 plastic containers (volume 40 dm^3, 96 seedlings per container) in a growth chamber (Weiss, Type 10 Sp/5 DU-Pi, Lindenstruth, Germany). The containers were filled with aerated nutrient solution containing a low concentration of nutrients (0.25 mM N and other essential nutrients in proportion to N) (Ingestad and Lund 1986). Air and root zone temperatures were adjusted to 4 °C and the seedlings were kept in continuous dim light (100 µmol m^{-2} s^{-1}) during the first 3 days. Thereafter, the containers were grouped in three blocks, each comprising four containers. Each block was then divided into two nutrient treatments, low nutrient availability (0.25 mM N; LN treatment) and high nutrient availability (2.5 mM N; HN treatment), with two containers per block per treatment. In the nutrient solutions (Ingestad and Lund 1986), nitrate and ammonium ions made up 58 and 42% of the total N, respectively. The concentrations of all other essential nutrients were supplied in proportion to the amount of N. The pH of the nutrient solutions was adjusted to 4.5–5.5 with 1 *N* NaOH or 1 *N* HCl. Electrical conductivity and pH of nutrient solutions were measured twice a week; nutrient solutions were added based on these measurements. In addition, the concentrations of NH_4-N and NO_3-N of the nutrient solutions were occasionally checked by flow injection analysis. The nutrient solutions were changed monthly. Daily and seasonal fluctuations in air and root zone temperature, relative air humidity and photosynthetic photon flux density were similar in all treatments. Air and root zone temperature and photoperiod were simulated, with slight modifications, to follow the mean weather and light conditions in Jokioinen (60°49′ N, 23°30′ E), based on meteorological data measured during 1961–90 (Finnish Meteorological Institute 1991) (Figure 1). In the simulated conditions, the length of a month was 26 days; and the total temperature sum that developed during the simulated growing season was 1044 d.d. (d.d. = degree days above a threshold of 5 °C) (Figure 1), which was the lower limit in the temperature sum zone of 1000–1300 d.d. in southern Finland between latitudes 60° and 64° N (Finnish Forest Research Institute 1998). Relative air humidity in the growth chamber was 60–80% in May and June, 60–90% in July and August and 70–90% in September and October, being lowest in the middle of the day and highest in the middle of the night. Air temperature and relative humidity in the chamber were recorded continuously by a thermohygrograph. Maximum daily photon flux density at the top of the seedlings was 300 µmol m^{-2} s^{-1} (PAR, Li-185B, Li-Cor, Lincoln, NE). To control root zone temperature, a water-filled plastic coil was placed in the bottom of each container. The coils were connected to cryostats (MGW, Lauda, Germany) that circulated water in the coil, thus maintaining the nutrient solutions at the desired temperature. The temperature of the nutrient solutions was measured daily with a digital thermometer (Sensotherm 100, Nürnberg, Germany).

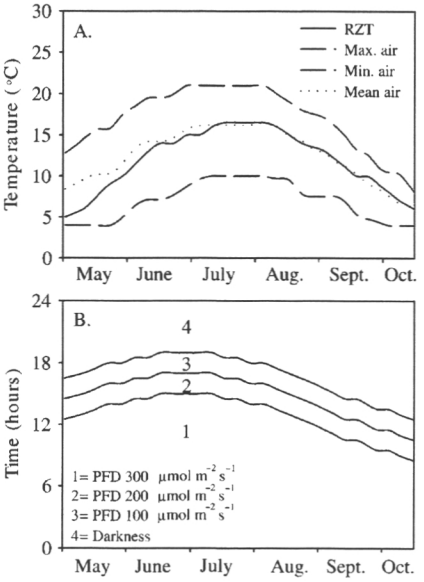

Figure 1. Maximum, minimum and mean daily air temperatures, and mean RZT (A) and length of the photoperiod (B) in the growth chamber during the experiment. Seedlings were kept in dim light for 2 h before (1 h at 100 µmol m^{-2} s^{-1} and 1 h at 200 µmol m^{-2} s^{-1}) and after (1 h at 200 µmol m^{-2} s^{-1} and 1 h at 100 µmol m^{-2} s^{-1}) a period of full illumination (300 µmol m^{-2} s^{-1}).

Growth and nitrogen analysis

Fresh mass of the seedlings was determined by weighing eight seedlings per container (48 seedlings per treatment) at the beginning of the experiment and nine times during the experiment. At each weighing, new seedlings were sampled for biomass analysis. After the current- and previous-year roots, needles and stems of eight sampled seedlings had been weighed separately, the organs were pooled and stored at –80 °C. Current-year roots were separated when the length of the new roots was > 1 cm. Dry mass was determined from a subsample that had been dried at 60 °C for 48 h. After drying, total N of the needle and stem samples was measured with a CHN-600 analyzer (Leco Co., St. Joseph, MI) and that of the root samples with a Leco CHN-900 analyzer. Specific absorption rate of N (SAR_N) was calculated as:

$$SAR_N = (M_2 - M_1)(\ln W_2 - \ln W_1)/(T_2 - T_1)(W_2 - W_1),$$

where M_1 and M_2 represent total N content in the plant and W_1 and W_2 represent fresh masses of roots at sampling times T_1 and T_2, respectively (Welbank 1962).

Isolation of plasma membranes from roots

Material for isolation of the plasma membrane was sampled 10 times during the experiment. Altogether eight seedlings were sampled systematically from each container and the roots of the sampled seedlings were excised and separated into current- and previous-year roots. Thereafter, roots from each container were pooled, frozen in liquid N and stored at –80 °C. Plasma membranes were isolated by a two-phase aqueous polymer technique (Widell 1987) from 10–15 g of frozen roots as described by Iivonen et al. (1999).

Assay of PM-ATPase activity and determination of membrane proteins

Activity of PM-ATPase was measured as described by Ryyppö et al. (1994) but with slight modifications. The assay is based on colorimetric measurement of inorganic phosphate released from ATP hydrolysis (Hodges and Leonard 1974). Activity was assayed in the presence of 5 mM NaN_3, 100 mM KNO_3 and 0.1 mM $(NH_4)_6Mo_7O_{24}$, and in the presence or absence of 100 μM $NaVO_4$ and 3 mM $MgSO_4$. The detergent used was 0.1% Brij 58. The PM-ATPase activity of the roots was measured at the actual RZT (hereafter called real PM-ATPase activity) and at 38 °C. The activity measured at 38 °C shows the maximal capacity of the enzyme (hereafter called potential PM-ATPase activity). The PM-ATPase activity was calculated per unit fresh mass of the roots and on the basis of total root fresh mass (hereafter called total activity). The amount of plasma membrane protein was estimated according to Bradford (1976).

SDS-PAGE and Western blotting

Plasma membrane samples were precipitated with 10% (w/v) trichloracetic acid for 15 min in an ice bath and pelleted by centrifugation at 4 °C. The pellets were suspended in buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 20 mM dithiothreitol, 10 mM EGTA, 0.02% (w/v) bromophenol blue, 1 mM PMSF and 50 μg ml^{-1} chymostatin. A 5 μg sample of plasma membrane proteins was subjected to SDS-PAGE (polyacrylamide gel electrophoresis) according to Laemmli (1970). The gel system consisted of a 4.8% stacking gel and an 8% resolving gel.

After gel electrophoresis, polypeptides were transferred electrophoretically to a nitrocellulose membrane (Hypond ECL, Amersham Pharmacia Biotech, Piscataway, NJ). The transfer buffer contained 25 mM Tris, 192 mM glycin and 20% (v/v) methanol. Transfer was performed at a constant current of 20 mA for 1 h at 4 °C. After transfer, the membrane was incubated for 1 h at room temperature in a blocking buffer (5% (w/v) defatted milk powder in TBS-T: 140 mM NaCl, 20 mM Tris-HCl (pH 7.6) and 0.1% (v/v) Tween 20). Thereafter, the membrane was washed with TBS-T for 1 × 15 min and 2 × 5 min. After washing, the membrane was incubated overnight at room temperature with rabbit antiserum (No. 721, used at a dilution of 1/10,000; a gift from Prof. Ramón Serrano, Universidad Polytechnica, Valencia, Spain) against the central domain of *Arabidopsis thaliana* (L.) Heynh. The antiserum was raised against fusion proteins containing amino acid sequences 340–650 of AHA3 (Pardo and Serrano 1989) and specifically recognized the same size of polypeptide (around 100 kD) from the plasma membranes of *A. thaliana* leaves and Scots pine roots (data not shown). After incubation, the membrane was washed again and then incubated with a secondary antibody (Anti-rabbit IgG, peroxidase-linked antibody from donkey, ECL, Amersham Pharmacia Biotech) for 1 h at room temperature. The secondary antibody was used at a dilution of 1/3000. Both antibodies were diluted in a buffer containing 1% (w/v) defatted milk powder, 140 mM NaCl and 20 mM Tris-HCl (pH 7.6). After incubation with secondary antibody, the membrane was washed again. Thereafter, the membrane was treated with ECL Western blotting reagents (Amersham Pharmacia Biotech) and then exposed to Kodak X-ray film for 1 min. The bands on the film were quantified by image analysis (SnapScan 1236, Agfa-Gevaert Group, Mortsel, Belgium).

Statistical analyses

Three-way analysis of variance was performed to test (1) the effects of nutrient treatment, age of the roots, block factor and sampling day on PM-ATPase activity and on the amount of plasma membrane protein (n = 6); (2) the amount of PM-ATPase, with age of the roots, nutrient treatment and sampling day as factors (n = 3); and (3) the effects of nutrient treatment, block factor and sampling time on root/shoot ratios, increment in N content and SAR_N (n = 6). Values of PM-ATPase activity, amount of PM-ATPase, protein concentration in the plasma membrane, N content and SAR_N in pooled samples for each container were used as independent observations. In the statis-

tical analysis, the mean values for root/shoot ratio of the sampled seedlings in each container were used as independent observations in the statistical analysis. When a significant interaction between sampling day and nutrient treatment or age of the roots was detected, the pairwise differences between treatment means were analyzed with Tukey's multiple range test. The analyses were conducted with SYSTAT 5.0 for Windows (SYSTAT, Evanston, IL).

Results

Growth of the seedlings

Initial fresh mass of the seedlings was 3.95 ± 0.20 g (± SD), and final fresh mass at the end of the growing season was 16.59 ± 2.30 g in the LN treatment and 29.74 ± 3.50 g in the HN treatment, indicating that the total fresh mass increment of HN seedlings was twice that of LN seedlings. In both nutrient treatments, the seedlings grew vigorously from June to the end of August (Figures 2A and 2B); thereafter, growth of the seedlings declined rapidly. Root growth started in early June, peaked at the end of August, declined in early September and again showed a small peak at the end of September. Compared with LN seedlings, root growth of HN seedlings was greater and continued longer at the end of the growing season (Figure 2B). In both nutrient treatments, root growth decreased at the end of July, when shoot growth was most intense, and accelerated subsequently. Compared with HN seedlings, LN seedlings allocated proportionally more biomass to root growth than to shoot growth after mid-July (Figures 2A and 2B, Table 1).

PM-ATPase activity per unit fresh mass of the roots

In both nutrient treatments, potential PM-ATPase activity (i.e., measured at 38 °C) was higher in current-year roots than in previous-year roots ($P < 0.001$) (Figure 3A). Potential PM-ATPase activities of current- and previous-year roots were higher in the HN treatment than in the LN treatment ($P < 0.001$ and $P = 0.04$, respectively). Seasonal changes in potential activity were similar in both nutrient treatments, with two exceptions: first, in the HN treatment, the potential activity of current-year roots was much higher at the end of June but decreased until mid-July; and second, in the HN treatment the decrease in potential activity started 2 weeks later, at the beginning of October.

Table 1. Root/shoot ratio (fresh mass) of Scots pine seedlings during the simulated second growing season. The seedlings were grown at low (0.25 mM N; LN treatment) or high (2.5 mM N; HN treatment) nutrient availability. Each value is the mean (± SE) of six replicates. Three-way ANOVA indicated a statistically significant interaction between sampling time and nutrient treatment. Within a row, values followed by different letters differ significantly from each other at $P < 0.05$ (Tukey's multiple range test).

Date	LN treatment	HN treatment
May 1	0.56 ± 0.02 a	0.56 ± 0.03 a
May 18	0.68 ± 0.04 a	0.68 ± 0.05 a
June 5	0.72 ± 0.07 a	0.62 ± 0.02 a
June 23	0.77 ± 0.04 a	0.77 ± 0.05 a
July 17	1.11 ± 0.06 a	0.97 ± 0.02 a
August 11	1.21 ± 0.04 a	0.88 ± 0.03 b
August 29	1.62 ± 0.05 a	0.97 ± 0.03 b
September 17	1.65 ± 0.04 a	1.05 ± 0.03 b
October 1	1.75 ± 0.08 a	1.17 ± 0.05 b
October 15	1.70 ± 0.13 a	1.22 ± 0.06 b

Real PM-ATPase activity (i.e., measured at the actual RZTs) of current-year roots ($P < 0.001$) and to some extent real activity of previous-year roots ($P = 0.01$) were higher in HN seedlings than in LN seedlings (Figure 3B). In both nutrient treatments, real activity was higher in current-year roots than in previous-year roots until the end of September ($P < 0.001$). In the HN treatment, real activity of current-year roots was high from the end of June to the end of August when RZT was 13.5–16.5 °C, decreased in mid-September and peaked at the end of September before it decreased rapidly. In the LN treatment, real activity of current-year roots was highest in mid-July but then decreased steadily showing a small peak at the end of September. Real activity of previous-year roots decreased earlier and at a higher rate in the LN treatment than in the HN treatment ($P < 0.001$ for the interaction between nutrient availability and sampling day), and in both HN and LN seedlings real activity was low in mid-October.

Total PM-ATPase activity of roots

We calculated total PM-ATPase activity to describe activity of the whole root system and that of current- and previous-

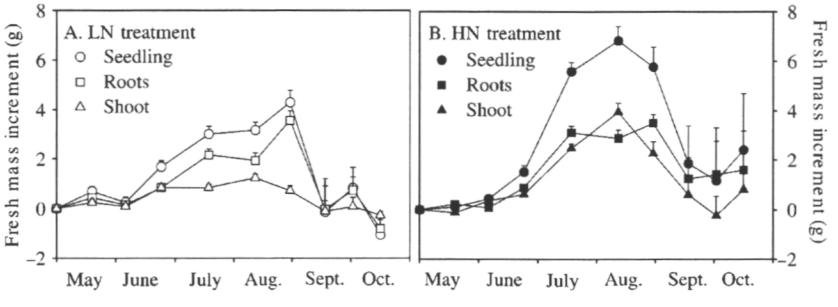

Figure 2. Fresh mass increments of shoot, roots and whole seedlings of Scots pine during the growing season at low (A; 0.25 mM N) or high (B; 2.5 mM N) nutrient availability. Each value represents the mean (+ SE) of six replicates.

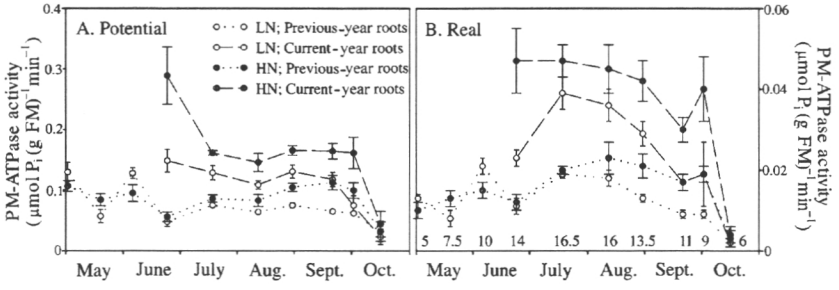

Figure 3. Potential (A) and real PM-ATPase activity (B) per unit fresh mass of current- and previous-year roots during the growing season. Seedlings were grown at low (0.25 mM N; LN) or high (2.5 mM N; HN) nutrient availability. Each value represents the mean (± SE) of six replicates, each with two replicate assays. Real activities were measured at actual RZTs, which are marked at the bottom of (B).

year roots separately. In both nutrient treatments, total real (i.e., measured at the actual RZTs) activity of current-year roots was significantly higher than that of previous-year roots ($P < 0.001$) (Figures 4A and 4B). Total real activity of all roots was low until the end of June; thereafter, it increased rapidly in the whole root system and in current-year roots and continued to increase until the end of August. It then decreased at the beginning of September and peaked again at the end of September before declining in October (Figures 4A and 4B). Although total real activity was higher in HN seedlings than in LN seedlings ($P < 0.001$), the trends over the growing season were similar. In both nutrient treatments, total real activity of previous-year roots was low and did not vary markedly during the growing season.

In both LN and HN seedlings, total potential activity (i.e., activity measured at 38 °C) was higher in current-year roots than in previous-year roots ($P < 0.001$; Figures 4C and 4D). Furthermore, total potential activity was significantly higher in HN seedlings than in LN seedlings ($P < 0.001$). In the LN treatment, total potential activity of the whole root system and of current-year roots increased until August and decreased thereafter (Figure 4C). In the HN treatment, total potential activity of the root system and of current-year roots increased from June to the end of September, and decreased rapidly thereafter (Figure 4D). In contrast to LN seedlings, total potential activity of previous-year roots of HN seedlings increased at the end of the growing season ($P < 0.001$ for the interaction between nutrient availability and sampling day).

Changes in amount of plasma membrane proteins and PM-ATPase

The amount of plasma membrane (PM) proteins was significantly higher in current-year than in previous-year roots ($P < 0.001$ in the LN treatment and $P = 0.004$ in the HN treatment; Figure 5). However, the amount of PM proteins in current-year roots was unaffected by nutrient treatment and sampling

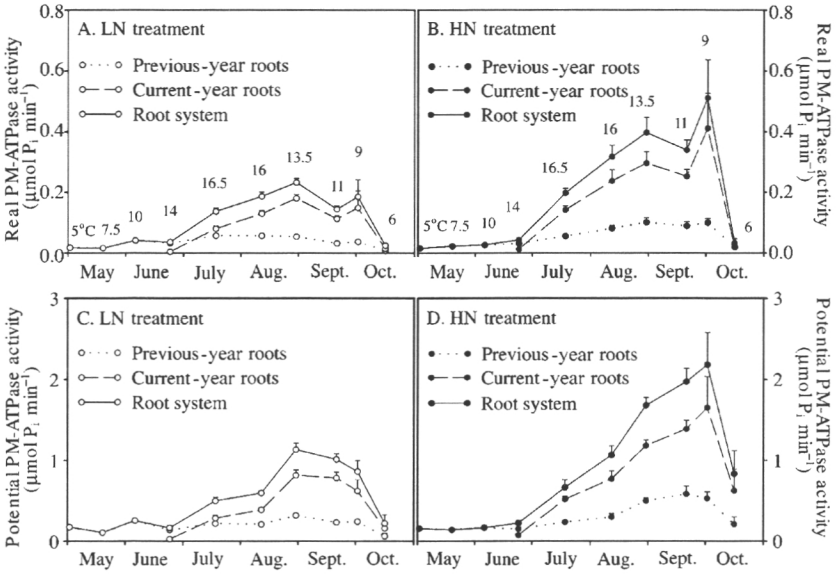

Figure 4. Total real (A, B) and potential (C, D) PM-ATPase activity of current-year roots, previous-year roots and the whole root system of Scots pine seedlings during the growing season. Seedlings were grown at low (0.25 mM N; LN) or high (2.5 mM N; HN) nutrient availability. Each value represents the mean (+ SE) of six replicates, each with two replicate assays. Real activities were measured at actual RZTs, which are marked on (A) and (B).

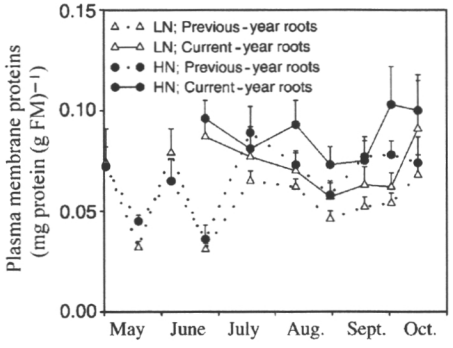

Figure 5. Amount of plasma membrane proteins in the current- and previous-year roots of Scots pine seedlings grown at low (0.25 mM N; LN) or high (2.5 mM N; HN) nutrient availability. Each value represents the mean (+ SE) of six replicates, each with two replicate assays.

day, whereas the amount of PM proteins in previous-year roots differed significantly between sampling days.

The amount of PM-ATPase was significantly higher in current-year roots than in previous-year roots in both LN ($P < 0.001$) and HN seedlings ($P = 0.005$) (Figures 6A and 6B). In previous-year roots, the amount of PM-ATPase varied over the growing season (Figure 6A) and in September it was significantly higher in HN seedlings than in LN seedlings ($P = 0.008$). In contrast, the amount of PM-ATPase in current-year roots was not affected by nutrient treatment and it remained in high amounts until mid-September when it began to decrease (Figure 6B).

Nitrogen net uptake

At the beginning of June, when growth of the seedlings started (Figures 2A and 2B), seedling N content increased rapidly (Figure 7A) and was much higher in the HN treatment than in the LN treatment ($P < 0.001$) (Figures 7C and 7D). In LN seedlings, N content increased until the end of August, whereas in HN seedlings N content increased until the end of the growing season (Figure 7A). At the end of the growing season, the N content of current- and previous-year roots increased markedly in the HN treatment (Figure 7D).

The specific absorption rate of N (SAR_N) was significantly higher in HN seedlings than in LN seedlings ($P < 0.001$) (Figure 7B). In seedlings in both nutrient treatments, SAR_N peaked in mid-July and thereafter decreased until mid-September. From mid-September to mid-October, SAR_N was steady but low in HN seedlings, whereas no N uptake was observed in LN seedlings.

Nitrogen concentrations of all tissues were higher in HN seedlings than in LN seedlings. In May, the N concentration of previous-year needles and stem decreased and that of the roots increased (Figures 7E and 7F). Thereafter, in both LN and HN seedlings, the N concentration of previous-year tissues remained relatively stable, whereas that of current-year tissues declined.

Discussion

Current-year roots are more active than previous-year roots

Both real and potential PM-ATPase activities and the amounts of PM-ATPase and total plasma membrane proteins were higher in current-year roots than in previous-year roots until the end of September, confirming the importance of current-year roots in nutrient uptake. The PM-ATPase is abundant in the root cap, pericycle, epidermal, endodermal and phloem companion cells (Parets-Soler et al. 1990, Jahn et al. 1998). The anatomy of the root changes during secondary growth (Kramer 1983), which affects the surface area of the plasma membrane accessible to the soil solution (Kamula et al. 1994), and therefore metabolic activity varies along the length of the root (Travis et al. 1979, Roldán et al. 1991, see

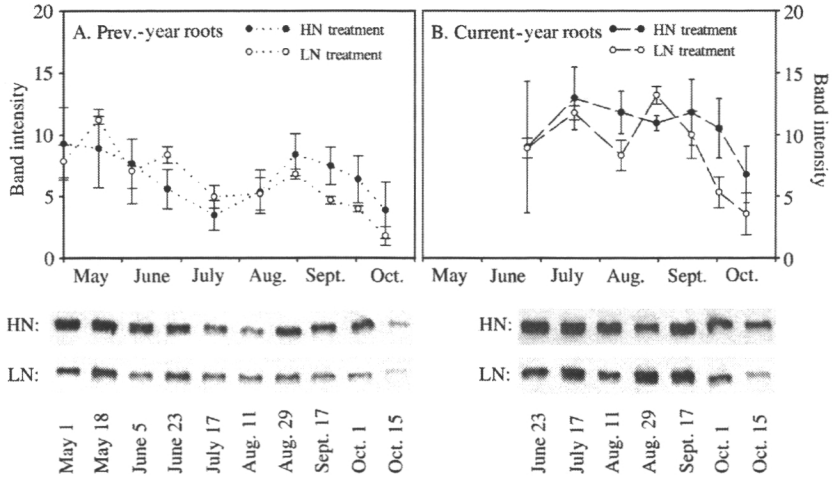

Figure 6. Changes in amount of root PM-ATPase in previous- (A) and current-year (B) roots of Scots pine seedlings grown at low (0.25 mM N; LN) or high (2.5 mM N; HN) nutrient availability. Upper panel: Each value represents the mean band intensity (± SE) of three replicates. Lower panel: Western blots of PM-ATPase ($M_r \approx 100$ kD) of previous- and current-year roots. Five μg of protein was applied to each well.

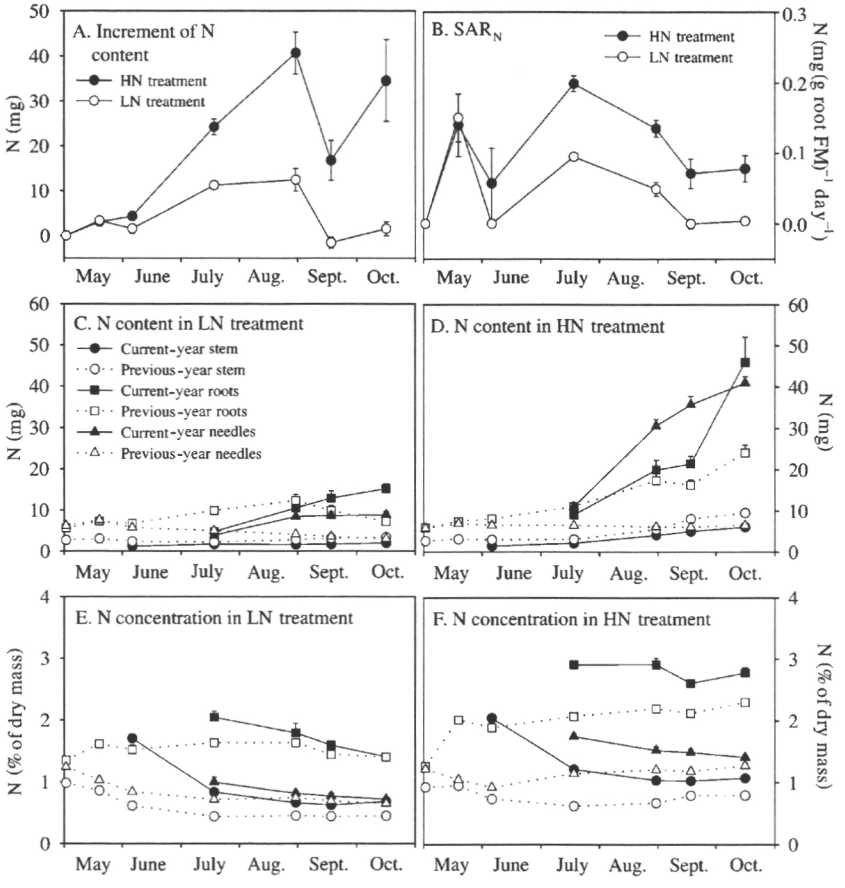

Figure 7. Increment of total N content (A), specific absorption rate of N (B), N content (C, D) and N concentration (E, F) in different plant parts of Scots pine seedlings during the growing season. Seedlings were grown at low (0.25 mM N; LN treatment) or high (2.5 mM N; HN treatment) nutrient availability. Each value represents the mean (± SE) of six replicates.

Marschner 1995). Because current-year roots are more active than previous-year roots, the seasonal variation in metabolic activity of the root system is strongly linked to the formation of new roots, and thus to the growth rhythm of the roots.

Activity of PM-ATPase per unit fresh mass of roots is acclimated to the size of the root system

Nutrient uptake rate is generally balanced by the demand of the plant for nutrients required for growth (White et al. 1991, Lainé et al. 1995); however, an accumulation of nutrients in the autumn is characteristic of conifer seedlings growing at high nutrient supply (van den Driessche 1985, Rikala and Repo 1997). We found a strong correlation between increment in N content and increment in total seedling biomass ($r = 0.74$ in the LN treatment and $r = 0.72$ in the HN treatment, data not shown). Total PM-ATPase activity of current-year roots was greatest at the end of the growing season, whereas PM-ATPase activity per unit fresh mass of current-year roots and SAR_N peaked earlier, indicating that a decrease in PM-ATPase activity per unit fresh mass of roots can be compensated by increasing the size of the root system. In response to nutrient limitation, pines usually allocate more biomass to the root system than to the aboveground organs, which leads to an increase in the root/shoot ratio (Squire et al. 1987, Gower et al. 1994, Iivonen et al. 1999). An increase in the root/shoot ratio was observed in LN seedlings in the present study (Table 1). Under nutrient-limited conditions, regulation of the root/shoot ratio seems to be of major importance in matching the metabolic activity of the root system to the requirement for nutrients.

High nutrient availability stimulates PM-ATPase activity

Between the end of June and mid-September, when RZT was over 9 °C, real PM-ATPase activity per unit fresh mass of current-year roots was 1.2–2 times higher in the HN treatment than in the LN treatment; however, this difference was not paralleled by a similar difference in the amount of the PM-ATPase. This finding indicates that high nutrient availability may regulate PM-ATPase by increasing its activity without obvious changes in the amount of PM-ATPase (cf. Roldán et al. 1991, Palmgren 1998). In several herbaceous species increases in root PM-ATPase activity (Kuiper et al. 1991) and microsomal ATPase activity (Kähr et al. 1977, Kuiper 1982,

Wignarajah et al. 1983) have been reported in response to an increase in external nutrient supply. Such changes may be associated with differential activation of isoforms of PM-ATPase that differ with respect to their kinetic parameters (Palmgren and Christensen 1994, Palmgren 1998) and might be specialized to function under different environmental conditions (Michelet et al. 1994).

Sensitivity of PM-ATPase activity to RZT is dependent on the stage of seedling development

In early September, shoot and root growth declined in both nutrient treatments, resulting in a low growth-related demand for nutrients. Simultaneously, real PM-ATPase activity of the root system and N net uptake decreased. At the end of September, root growth was again accelerated in both HN and LN seedlings. At the same time, real PM-ATPase activity and net uptake of N increased, although RZT decreased from 11 to 9 °C. Earlier studies have shown that PM-ATPase activity is dependent on RZT in spring (Ryyppö et al. 1998, Iivonen et al. 1999). In contrast, we found that, although total real PM-ATPase activity was dependent on RZT in spring, this temperature dependence disappeared in August and total real PM-ATPase activity was highest at the end of the growing season when RZT was decreasing (Figures 4A and 4B). Our data strongly suggest that root growth (Iivonen et al. 2001) and sensitivity of root PM-ATPase to RZT vary during the growing season and are dependent on the developmental stage of the seedling. In October, when RZT decreased from 9 to 6 °C, both the real and potential PM-ATPase activities decreased rapidly. This decrease was not linked to changes in PM proteins, which showed no change at the end of the growing season. We suggest that both the amount and activity of PM-ATPase decreased to basal values because of a reduction in growth-related demand for ion uptake and transport.

High nutrient availability extends growing period of roots

During the growing season, total net uptake of N was 5.3 times higher in HN seedlings (133.0 ± 22.7 (SD) mg) than in LN seedlings (25.1 ± 7.5 mg), whereas increment in fresh mass of seedlings only doubled in the HN treatment (25.7 ± 3.5 g (SD)) compared with the LN treatment (12.75 ± 2.4 g). During the growing season, N concentration of current-year needles decreased from 1.7 to 1.4% in the HN treatment and from 1.0 to 0.7% in the LN treatment. An adequate range of N concentration for shoot growth of 1-year-old Scots pine seedlings is 1.6–2.0% (Rikala 1982). Thus, N deficiency limited growth in LN seedlings but not in HN seedlings.

In May, when PM-ATPase activity and N net uptake of roots were low, the demand for N for growth was met by retranslocation from previous-year needles and the stem. At the end of the growing season, real and potential PM-ATPase activities were higher in HN seedlings than in LN seedlings, enabling roots of HN seedlings to grow for a longer period and to take up N until the end of the growing season. In contrast, only a very small amount of N was taken up after August in LN seedlings. Studies with Sitka spruce (*Picea sitchensis* (Bong.)) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) have shown that fine root production, in particular, increases in response to high nutrient availability (Coutts and Philipson 1977, Friend et al. 1990). Although previous-year roots have a minor role in N uptake, both the amount and activity of PM-ATPase in previous-year roots were stimulated by the HN treatment from the end of August to the beginning of October. This stimulation might be associated with root branching, which is increased when nutrient availability is high (Coutts and Philipson 1977, Rikala and Huurinainen 1990), leading to increases in the amount and activity of PM-ATPase in segments of previous-year roots where the lateral roots develop (Travis et al. 1979).

In conclusion, PM-ATPase activity of the root system and N net uptake of Scots pine seedlings varied considerably during the growing season, and were highly dependent on formation of current-year roots. Activity of PM-ATPase of the root system was low in spring and early summer and peaked at the end of the growing season when RZTs were decreasing. At RZTs below 9 °C, PM-ATPase activity of the root system was low, but no clear dependence of metabolic activity on temperature was found at higher RZTs. High nutrient availability promoted PM-ATPase activity of current- and previous-year roots, allocation of biomass to aboveground organs and also extended the growing period of the seedlings. Stimulation of PM-ATPase activity by high nutrient availability could not be explained by increased amount of the enzyme, indicating additional regulation of PM-ATPase activity under conditions of high nutrient availability.

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