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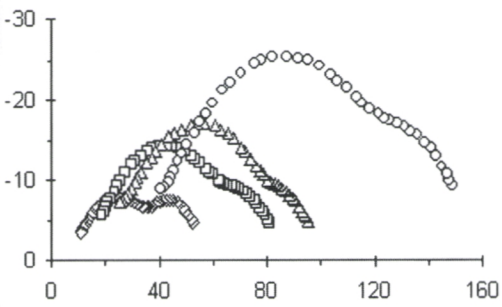
COLD ACCLIMATION IN SCOTS PINE

D.Sc. (Agr. and For.) thesis

Gang Zhang



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COLD ACCLIMATION IN SCOTS PINE

Gang Zhang

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Forestry of the University of Joensuu, for public criticism in Auditorium MA 155 of the Borealis Building, Yliopistokatu 7, Joensuu, on November 9th 2001, at 12 o'clock noon.

SUONENJOEN TUTKIMUSASEMA
SUONENJOKI RESEARCH STATION

Supervisors: Dr. Tapani Repo
Joensuu Research Centre
Finnish Forest Research Institute, Finland

Dr. Taneli Kolström
Faculty of Forestry
University of Joensuu, Finland

Thesis supervisors:
Dr. Aija Ryyppö
Faculty of Forestry
University of Joensuu, Finland

Dr. Risto Rikala
Suonenjoki Research Station
Finnish Forest Research Institute, Finland

Reviewers: Professor Heikki Hänninen
Department of Ecology and Systematics
University of Helsinki, Finland

Dr. Marja-Liisa Sutinen
Kolari Research Station
Finnish Forest Research Institute, Finland

Opponent: Professor Olavi Junttila
Department of Applied Biology
University of Helsinki, Finland

Available at: The Finnish Forest Research Institute
Library
P.O. Box 18
FIN-01301 Vantaa

Phone: +358 9 857 051
Fax: +358 9 8570 5582
E-mail: kirjasto@metla.fi

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ABSTRACT

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This thesis presents the results of studies concerning frost hardening in Scots pine (*Pinus sylvestris* L.). The studies were based on an 8-year field provenance trial of Scots pine established in central Finland (62°39'N, 27°03'E, 130 m a.s.l.) with six origins ranging from Estonia to northern Finland, and on second-year Scots pine seedlings from central Finnish origin (61°42'N, 24°56'E, 160 m a.s.l.) in growth chambers. The frost hardiness of the stems, needles, buds and roots of Scots pine saplings and seedlings were assessed in controlled freezing tests by means of the visual scoring of the damage, electrolyte leakage and electrical impedance methods related to organ. The electrical impedance spectroscopy parameters, the dry matter content, the maximum photochemical efficiency of photosystem II (F_v/F_m) and the sugar concentration of the non-frost-exposed samples were subjected to study. The main aim of the present study is, then, to examine cold acclimation in Scots pine. The particular objectives of the study are to find a simple and fast method for assessing frost hardiness and to test whether the frost hardiness of different organs is an additive property of the environmental factors and whether a stationary level of frost hardiness prevails for those environmental factors.

The pattern of growth cessation tended to follow the latitude of origin, i.e. growth ceased in the northernmost origin first and the southernmost one last. Both the stems and needles of the northern origin hardened earlier than the southern ones, but the differences in hardiness disappeared as hardening progressed. Growth cessation and initial hardening to –15°C in the stems and needles were clearly correlated at the provenance level. The electrical impedance parameters relaxation time τ_1 and the intracellular resistance r_i of stems increased with increasing frost hardiness. At a given time, it was generally possible to predict the frost hardiness with τ_1 and r_i, even though the relationship between the frost hardiness and these two parameters changed during the autumn season. The highest coefficient of determination between frost hardiness and τ_1 was 0.95 in September and was predicted with an accuracy of ±2.0°C. In current-year needles, according to the cell membrane time constant τ_m, extracellular resistance r_e and β-coefficient (a factor in Model-A controlling spectrum skewness and impedance locus centre depression) there was a clear gradation between origins in the prehardening phase in August, lasting until mid-September. From the end of September significant differences were observed in the intracellular resistance r_i of needles according to their origins and corresponding with the differences in their hardening patterns.

There was a clear difference in hardiness between the organs by the end of the photoperiod and temperature treatments but no difference between the treatments in the stems, needles and buds. In the roots a stationary level of frost hardiness was reached, whereas in the case of the stems and needles the frost hardiness asymptotically approached the respective stationary level. This proves that the discrete stationary level of frost hardiness according to photoperiod and temperature may be found during cold acclimation. Very little support was found for the concept of additive effects by photoperiod and temperature. In the initial phase, the hardening of needles was driven predominantly by the photoperiod, while the hardening of stems was driven mainly by temperature. The hardening of roots was mainly driven by temperature throughout the treatments. The sugar

concentration in the different organs followed the sequence needles > stems > roots, which also matched the levels of frost hardiness. The dry matter content of the stems, needles and roots increased during hardening. The performance of the dry matter content in stems, needles and roots differed in Scots pine saplings and seedlings. It is suggested that the dry matter content of the stems, needles and roots, and F_v/F_m of non-frost-exposed needles are not promising predictors of frost hardiness.

Keywords: Dry matter content, electrolyte leakage, frost hardiness, F_v/F_m, growth cessation, impedance spectroscopy, modelling, photoperiod, *Pinus sylvestris*, sugar concentration, temperature

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Author's address: Gang Zhang, University of Joensuu, Faculty of Forestry, P.O. Box 111, FIN-80101 Joensuu, Finland. Tel: +358 13 251 4442. Fax: +358 13 251 4444.
E-mail: gang.zhang@forest.joensuu.fi

Author's current address: Gang Zhang, Finnish Forest Research Institute, Suonenjoki Research Station, Juntintie 40, FIN-77600 Suonenjoki, Finland. Tel: +358 17 513 811. Fax: +358 17 513 068. E-mail: gang.zhang@metla.fi

PREFACE

This study has been carried out at the Suonenjoki Research Station of the Finnish Forest Research Institute and at the Faculty of Forestry of the University of Joensuu. The work was financed by the University of Joensuu, the Finnish Forest Research Institute, the Academy of Finland Research Council for Environmental and Natural Resources, and the China Scholarship Council. I gratefully acknowledge the support provided by all of these institutions.

I am particularly grateful for the guidance given by my general research supervisors Dr. Tapani Repo and Dr. Taneli Kolström. Dr. Taneli Kolström kindly recommended me to study at the Faculty of Forestry in Joensuu in 1998 at a time when he was both a professor there and also the Dean of the Faculty, and he contributed greatly by organizing the financing of my doctoral studies. Dr. Tapani Repo gave me an invaluable introduction to the field of frost hardiness, and always had time to guide and advise me during the course of my studies. Both have enabled me to realise my dream of becoming a Doctor in the field of Forestry. My warmest thanks go to Dr. Aija Ryyppö and Dr. Risto Rikala, who have acted as my thesis supervisors, for their unselfish guidance and encouraging comments. I am also grateful to the co-authors of the articles, Mr. Martti Vuorinen and Dr. Elina Vapaavuori, for their valuable and always pleasant co-operation.

I am sincerely indebted to Professor Paavo Pelkonen, the present Dean of the Faculty of Forestry, and to Dr. Heikki Smolander, the director of the Suonenjoki Research Station. They have generously provided me with excellent facilities for carrying out this work and they have supported me throughout its execution.

My sincere thanks are also to the official reviewers of this thesis, Professor Heikki Hänninen and Dr. Marja-Liisa Sutinen, for their constructive criticism and their careful work in reviewing the thesis, and to also Dr. John A Stotesbury for revising the English language of the manuscript.

I should also like very much to thank the whole staff of the Faculty of Forestry and of the Suonenjoki Research Station for all their friendly advice and assistance during the course of my studies and research. I am especially grateful to Professor Seppo Kellomäki, Professor Timo Pukkala, Mr. Markku Ropo, Dr. Jaana Luoranen, Dr. Marja Poteri and Mr. Pekka Voipio for their help. My particular thanks also go to Ms. Anna-Maija Väänänen, Ms. Eija Lappalainen, Mr. Ossi Muuronen, Ms. Asta Laurila, Ms. Hanna Kankkunen and Ms. Eeva Vehviläinen for their valuable assistance during the course of the experiments.

Finally, I would like to thank my wife Hong-Bin Ji and my son Chi Zhang for their support, encouragement and patience.

Joensuu, October 2001 Gang Zhang

LIST OF ARTICLES

I Repo, T., Zhang, G., Ryyppö, A., Rikala, R. and Vuorinen, M. (2000). The relation between growth cessation and frost hardening in Scots pines of different origins. Trees 14: 456-464.

II Repo, T., Zhang, G., Ryyppö, A. and Rikala, R. (2000). The electrical impedance spectroscopy of Scots pine (*Pinus sylvestris* L.) shoots in relation to cold acclimation. Journal of Experimental Botany 51: 2095-2107.

III Zhang, G., Ryyppö, A. and Repo, T. The electrical impedance spectroscopy of Scots pine needles during cold acclimation. Submitted to Physiologia Plantarum.

IV Zhang, G., Ryyppö, A., Vapaavuori, E. and Repo, T. Cold acclimation in Scots pine: a test of additive response and stationarity of frost hardiness by photoperiod and temperature. Submitted to the Canadian Journal of Forest Research.

In Studies I, II and III Gang Zhang performed all of the measurements and most of the data analyses under the supervision of Dr. Repo, Dr. Ryyppö and Dr. Rikala. Dr. Repo and Gang Zhang were mainly responsible for the writing of articles I and II, and Gang Zhang was solely responsible for writing article III. In the case of Study IV, Gang Zhang carried out the planning of the experiment under the supervision of Dr. Repo and Dr. Ryyppö. He also performed all of the measurements apart from the measurement of sugar concentration, he analysed the data, and he was responsible for writing the article. The co-authors had detailed discussions together in connection with each of the articles.

CONTENTS

SYMBOLS AND ABBREVIATIONS

$\Delta \hat{R}_P$	increase in stationary frost hardiness induced by photoperiod	°C
$\Delta \hat{R}_T$	increase in stationary frost hardiness induced by temperature	°C
$\hat{R}_{min}$	minimum level of frost hardiness	°C
$\hat{R}(t)$	stationary frost hardiness	°C
α , β	coefficients specific for organs (between 0 and 1) in equation [1]	
β	a factor in Model-A controlling spectrum skewness and impedance locus centre depression	
ψ_1	distribution coefficient of relaxation time τ_1	
τ_1	relaxation time	s
$\tau_m=R_3 \bullet C_m$	membrane time constant in Model-A	s
C_m	specific membrance capacitance	$\mu F/cm^2$
C_R	hardening competence (between 0 and 1)	
DCE	distributed circuit element	
DM	dry matter content: (dry weight/fresh weight)•100	%
EIS	electrical impedance spectroscopy	
FH	frost hardiness	°C
F_m	maximum fluorescence	
F_o	ground fluorescence	
F_v	variable fluorescence	
F_v/F_m	maximum photochemical efficiency of photosystem II	
LDLT	long photoperiod with a low temperature	
PS II	photosystem II	
r_1, r_2	specific resistances in the double distributed circuit element	
R^2	coefficient of determination	
R_3	specific membrane resistance	Ωcm^2
r_e	specific extracellular resistance	Ωm
r_i	specific intracellular resistance	Ωm
SDHT	short photoperiod with a high temperature	
SDLT	short photoperiod with a low temperature	

1 INTRODUCTION

1.1 General background of the study

Forest trees in boreal zones face four seasonal changes. It has been suggested that there are four phases of annual growth cycle of trees, i.e. active growth, lignification, rest and quiescence (Fuchigami et al. 1982, Kellomäki et al. 1992). Correspondingly, the annual cycle of frost hardiness can be divided into four periods, i.e. susceptible, hardening, maximal hardiness, and dehardening (Fig.1). These annual growth cycles are closely timed with the seasonal changes in the local climate (Howe et al. 1999). During the annual growth cycle, cold acclimation is an important phase during which the trees encounter great environmental changes. After the growing period, plants must survive the cold season, during which the temperature changes from a high level to a low level, and then below zero; for example, the temperature may fall to as low as –40°C and –60°C (Hurme 2000); and the photoperiod will shift from that of long days to that of very short days. The physiological, physiochemical, biochemical and anatomical characteristics of trees vary remarkably so that they can acclimate to the big changes in environmental conditions and be properly ready to meet the harsh winter.

Studies on cold acclimation have received a great deal of attention. Cold acclimation is concerned with the way in which frost hardiness synchronizes with the changes in the environmental conditions. In the synchronization of the phenology and frost hardiness of native origins with the weather conditions of the local growing site, two adaptive traits, the timing of growth cessation and the initiation of frost hardening, play key roles. In many tree species growth cessation is regarded as a prerequisite for cold acclimation (Weiser 1970, Siminovitch 1981, Junttila and Kaurin 1990). This synchronization is affected by certain environmental factors, including temperature, photoperiod, water stress, light and the supply of nutrients (Tumanov and Krasavtsev 1959, Aronsson and Eliasson 1970, Christersson 1978, Levitt 1980, Repo 1993, Beuker et al. 1998).

It has been indicated that, as autumn approaches, freezing tolerance develops, first induced by the short days and then by the first autumn frost (Weiser 1970). The shortening photoperiod is associated with the cessation of shoot elongation, bud formation and dormancy, which in turn influence the frost hardiness process (Arnott and Mitchell 1982,

Glerum 1985). The short day induction of the frost hardiness process is found in some conifer species, namely *Pinus sylvestris* L., *Picea abies* (L.) Karst. (Aronsson 1975, Christersson 1978), *Pseudotsuga menziesii* (Mirb.) Franco (van den Driessche 1969a, Timmis and Worrall 1975), *Pinus radiata* D. Don (Greer and Warrington 1982), *Picea mariana* (Mill.) B.S.P. (black spruce) (D'Aoust and Cameron 1982, Colombo 1986), and *Picea glauca* (Moench) Voss (white spruce) (Bigras and D'Aoust 1990, 1992). The influence in the autumn of low temperatures on hardiness development has been reported for many plants (McGuire and Flint 1962, Hamilton 1973, Ormrod and Layne 1974). Low temperature is an important factor in the development of hardiness and it is also a prerequisite for the maintenance of that hardiness. Acclimation in the autumn appears to involve the kind of metabolic processes that are favoured by low temperatures (Aronsson and Eliasson 1970).

Since the early work done by Heber and Santarius (1964) it is generally accepted that the membranes are the primary sites of frost damage in the cell. At present, the plasma membrane and the tonoplast are assumed to be the most frost-sensitive components of a cell (Ziegler and Kandler 1980, Steponkus et al. 1990, Leborgne et al. 1992). As temperatures drop below freezing, ice formation is generally initiated in the extracellular spaces of plants and, because the chemical potential of ice is less than that of liquid water, there is movement of unfrozen water from inside the cell to the extracellular spaces, where it freezes (Thomashow 2001). Trees native to the boreal zone become capable of tolerating the stress caused by extracellular ice formation through a complicated physiological process of frost hardening (Ryyppö et al. 1998). Tolerance to cold-induced dehydration is a key survival strategy in most cold-hardy plant species (Lee and Chen 1993).

The traditional methods of frost hardiness assessment, including the visual scoring of damage, the electrolyte leakage test, the measurement of chlorophyll fluorescence, differential thermal analysis, and electrical impedance analysis, require a control-freezing test conducted at several different freezing temperatures. Thus, the frost hardiness assessment is time-consuming, and it requires expensive equipment and also a considerable amount of material. Researchers have attempted to find a fast and easy method for the determination of frost hardiness in plants, and it has been found that several of the equivalent circuit parameters of the distributed model in an electrical impedance analysis of the stems of Scots pine undergo seasonal variation (Repo et al. 1995, 1997).

Clear relationships between the changes in some of the parameters measured by the non-frost-exposed samples and frost hardiness assessed by controlled freezing test have been found in several plant species. The highest correlations were previously observed between the intracellular resistance r_i and the frost hardiness (Repo et al. 1995, 1997, Väinölä and Repo 2000).

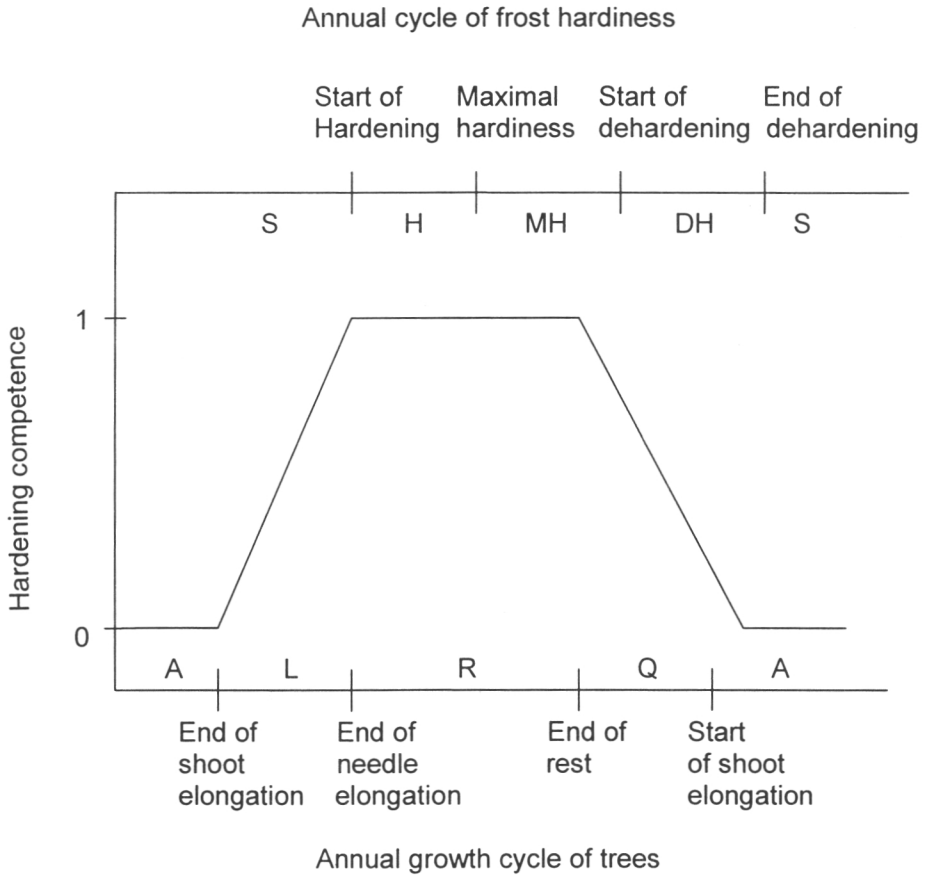

Figure 1. The annual growth cycle of trees and the annual cycle of frost hardiness in trees (schematically). The changes in their hardening competence C_R. A=active growth, L=lignification, R=rest, Q=quiescence and S=susceptible, H=hardening, MH=maximal hardiness, DH=dehardening (cf. Leinonen 1997).

The mathematical models concerned with cold acclimation are valuable for predicting and explaining the changes and development of frost hardiness in quantitative ways. All of

these models use environmental variables as input, either as temperature alone or as a combination of temperature and photoperiod (Leinonen 1997). The modelling approaches differ greatly from each other. Basically, two main categories of models can be distinguished, i.e. regression models and dynamic models. The regression models have been developed in relation to the frost hardiness of trees by estimating the relationship that exists between selected environmental variables and the measured frost hardiness. The dynamic models, for their part, can realistically describe the actual development of frost hardiness in relation to the environment. However, models need to be proved through experimentation and under natural conditions before they can be applied in practice. Any models gaining approval will then enable researchers to make progress towards a quantitative understanding of frost hardiness and the factors that contribute to it.

1.2 Aims of the study

The general aim of this study is to examine cold acclimation in Scots pine (*Pinus sylvestris* L.). More specifically, the following objectives were included:

1) To examine the cessation of shoot and needle elongation and of the diameter growth of the stem in relation to the frost hardening of Scots pine saplings in a provenance field trial (I);
2) To compare the relationship between the frost hardiness (FH) of stems and needles assessed by means of controlled freezing tests with the electrical impedance spectroscopy (EIS) parameters of stems (II, IV) and needles (III) not artificially exposed to frost (termed non-frost-exposed samples)
3) To compare the relationship existing between the FH of Scots pine needles assessed by means of controlled freezing tests and the maximum photochemical efficiency of photosystem II (F_v/F_m) of the non-frost-exposed needles (IV);
4) To test the relationship existing between the FH of Scots pine stems, needles and roots, assessed by means of controlled freezing tests and the dry matter (DM) content (II, III, IV) and sugar concentration (IV) of the non-frost-exposed samples;
5) To study the FH of buds, stems, needles and roots during cold acclimation in order to test the following hypotheses: (1) the cold acclimation in different organs is an additive property driven by photoperiod and temperature; (2) the FH of different organs will attain

a discrete stationary level corresponding to any given photoperiod and temperature; and (3) the frost hardiness of the different organs will respond similarly to the environmental factors or not (IV).

2. LITERATURE REVIEW

2.1 Frost hardiness in trees

Frost hardiness is the freezing temperature which a plant can sustain without being damaged (Glerum 1985, Kramer et al. 2000). The frost hardiness of trees changes seasonally. Morphology, developmental and physiological processes, as well as complex environmental interactions, are all involved in these changes (Guy 1990). Hardening starts as a response to declining day length and temperature, but their exact roles in the process are not thoroughly understood (Koski and Sievänen 1985, Hänninen et al. 1990).

The natural range of Scots pine is the widest among the pine species, from Spain (38°N) in the south to northern Finland (68°N), and from western Scotland (6°W) to eastern Siberia (135°E) (Mirov 1967). In Scots pine the severity and seasonal occurrence of frost varies geographically (Repo et al. 2001). This means that survival depends on trees possessing a certain degree of frost hardiness throughout the year. After growth cessation, frost hardening is initiated and trees enter dormancy, a period when growth is restricted by internal physiological and/or external environmental factors. After terminal bud set, the shortening of the photoperiod initiates frost hardening in the needles, which is further enhanced by the declining air temperature (Leinonen et al. 1995, Hurme et al. 1997, Rikala and Repo 1997).

Trees from northern latitudes acclimate faster and earlier than those from more southerly ones. Latitudinal variation in frost hardiness was found across the range of populations of first-year seedlings and also second-year seedlings, as well as in older trees (Hurme et al. 1997). These findings have been reported previously for several tree species, e.g., *Pinus sylvestris* L. (Toivonen et al. 1991, Aho 1994), *Pinus contorta* Dougl. ex. Loud. (Jonsson et al. 1981), and *Picea glauca* (Moench) Voss (Simpson 1994), confirming the importance for adaptation. Compared with coastal clones, continental clones started

hardening earlier, and a continental clone will proceed through hardening more rapidly at a given temperature (Ögren 1999a,b). A cross between a continental and coastal clone was found to be intermediate in the timing of its hardening. For instance, coastal populations of *Picea sitchensis* (Bong.) Carriére and *Tsuga heterophylla* (Raf.) Sarg. from Washington (Seattle) are less frost hardy than those from Alaska (Juneau), and inland populations of *Tsuga heterophylla*, *Thuja plicata* Donn ex D. Don and *Pseudotsuga menziesii* are more frost hardy than populations from the Pacific coast, while *P. menziesii* var. *glauca* (Mayr) Franco from the Rocky Mountains is substantially hardier (with a foliar resistance of –70°C) than coastal populations of *P. menziesii* var. *menziesii* (–20°C) (Sakai and Weiser 1973).

Differences in frost hardiness within the same tree occur between the various tissues, such as phloem, cambium and xylem, and also between various organs, such as needles, buds and bark (Karlsson and Palta 1999). In the hardiest species, leaves are hardier than overwintering buds, whereas less hardy species tend to have buds that are hardier than their leaves. Woody stem tissues are generally more resistant to frost than the individual leaves and buds, and (except during the growth period) the cambial tissues are more resistant than the other stem tissues. In Scots pine, under controlled conditions, the needles start to harden earlier than the stems (Ryyppö et al. 1998). Furthermore, Scots pine stems harden more slowly than needles under artificial conditions (Ryyppö et al. 1998).

In Scots pine, in the case of the first-year seedlings, northern origins initiate cold hardening earlier than more southerly ones (Dormling et al. 1977, Nilsson and Eriksson 1986, Aho and Pulkkinen 1991, Toivonen et al. 1991, Aho 1994, Sundblad and Andersson 1995, Hurme et al. 1997), but the altitude of the origin has no effect on the frost hardiness of Swedish and Finnish provenances (Andersson 1992, Sundblad and Andersson 1995). In older seedlings, shoot elongation ceases early at around midsummer, long before the cessation of needle elongation and well before the initiation of hardening (Repo 1992). Needle elongation continues longer than shoot elongation, lasting even up to the initial stages of frost hardening (Dormling et al. 1977, Rikala and Huurinainen 1990, Repo 1992, Rikala and Repo 1997). In general, differences in the timing of hardening in the early autumn are mainly indications of origin, but climatic factors also have some effect.

Although much is known about the factors affecting shoot frost hardiness, comparatively little is known about the factors affecting root frost hardiness (Colombo et al.

1995). The roots of trees seldom attain the same degree of frost hardiness as the above-ground parts (Smit-Spinks et al. 1985, Coleman et al. 1992, Ryyppö et al. 1998, Sutinen et al. 1998), nor are they usually exposed to temperatures as low as shoots are during frost hardening. However, the extent to which these differences in tissue frost hardiness are the result of structural, biochemical or environmental effects is not well understood.

2.2 Cold acclimation

Cold acclimation refers to the natural development of hardiness in the autumn and early winter (Fuchigami et al. 1982) (Fig. 1). The development of frost hardiness can be divided into three acclimation stages (Valkonen et al. 1990). The first stage of acclimation is induced by a short photoperiod (Aronsson 1975, Christersson 1978) and favoured by warm temperatures (Tumanov and Krasavtsev 1959, Howell and Weiser 1970, Fuchigami et al. 1971), and by only a few degrees' increase in frost hardiness. Growth cessation is a prerequisite for the first stage (Howell and Weiser 1970, Fuchigami et al. 1971). The second stage of acclimation is induced by low temperatures (Tumanov 1967, Weiser 1970, Proebsting 1978) and frost (Tumanov and Krasavtsev 1959, Howell and Weiser 1970). Howell and Weiser (1970) have postulated that short days and frost regulate different and independent endogenous acclimation processes. In this stage, trees usually become sufficiently frost hardy to tolerate the lowest temperatures of the year. It has been reported that there is also a third stage of acclimation which is induced by prolonged exposure to temperatures between –30°C and –50°C. During this stage, trees can resist temperatures as low as –196°C (Tumanov and Krasavtsev 1959).

Research reports indicate that cold acclimation is induced in pine and spruce seedlings by the photoperiod and that the process is stronger and more rapid at lower temperatures (Aronsson et al. 1976). Contradictory results have been obtained concerning the effects of photoperiod and temperature on the cold acclimation of conifer roots. Both an extended photoperiod and a warm temperature interfered with root acclimation to cold. Seasonally short days and a near-freezing temperature were necessary to produce the maximum rates of cold acclimation in roots (Johnson and Havis 1977). The short day exposure of plants induces root cold acclimation in the *Picea* and *Potentilla* species (Johnson and Havis 1977). However, most research studies have reported on the influence of low temperatures on root

acclimation (Bigras et al. 2001). The termination of root growth and the onset of root hardening in trees are mainly determined by a falling root zone temperature in autumn (Weiser 1970, Smit-Spinks et al. 1985, Bigras and D'Aoust 1992, 1993, Coleman et al. 1992, Ryyppö et al. 1998, Sutinen et al. 1998). In Scots pine seedlings, short day treatments do not induce any substantial hardening in roots (Smit-Spinks et al. 1985, Ryyppö et al. 1998). As far as root acclimation is concerned, the extent of root hardiness, the importance of photoperiod and thermoperiod in the control of acclimation, the relationship between root growth and hardiness, and the interrelationship of roots and shoots in whole plant hardiness are not well known (Smit-Spinks et al. 1985).

Siminovitch et al (1967) have suggested that endogenous seasonal rhythms, rather than low temperature *per se*, are the controlling factors in hardiness. Cold acclimation and dormancy are vital processes in plant survival, and both are affected by the same environmental factors. It is possible that the endogenous factors influencing these processes may also be similar.

The physiological and biochemical changes that result in cold acclimation have been under investigation in many laboratories (Smith 1968, Alden and Hermann 1971, Levitt 1972, 1980, Steponkus 1984, Sakai and Larcher 1987, Sutinen 1992, Hincha et al. 1996, 1997, Bertrand et al. 1999). Fundamental changes in the biochemical composition of woody plants are observed during autumn acclimation (Sutinen 1992, Bertrand et al. 1999). It is well established that cellular membranes such as the plasma membrane (Steponkus 1984) and thylakoids (Hincha and Schmitt 1992, Hincha et al. 1996) are the primary targets of freezing injury in plant cells. The plasma membrane assumes a central role. During cold acclimation, changes in the lipid composition of membranes (Lynch and Steponkus 1987, Sutinen 1992, Steponkus et al. 1993) have been observed and have been linked to an increase in frost hardiness. The water permeability of the plasma membrane, the concentrations of sugars, organic acids, and amino acids all increase during cold acclimation (Alden and Hermann 1971, Levitt 1972, 1980). The binding of the proteins to the membrane surface is an essential step in the process of protection. This binding brings hydrophobic domains on the protein surface into contact with the membrane. This hydrophobic interaction between protein and membrane may then lead to changes in the physical properties of the membrane that result in cryoprotection (Hincha et al. 1997).

2.3 Growth cessation during cold acclimation

Previous research studies show that the cessation of shoot and needle elongation, cambial growth, the lignification of new shoots and bud formation are all prerequisites for the onset of shoot dormancy and cold acclimation (Dietrichson 1961, van den Driessche 1969b, Weiser 1970). In boreal regions the timing of growth cessation is an essential aspect of the climatic adaptation of trees (Partanen and Beuker 1999). Oleksyn et al. (1992) have confirmed that the timing of growth cessation is regulated by both genotypic and environmental (photoperiodic) control. Those plants that stop growing earlier typically develop earlier frost hardiness (e.g. Cannell et al. 1987, Hurme et al. 1997). The ability of a species to synchronize its growth and dormant phases with periods of favourable and unfavourable climatic conditions is of paramount importance for its success in a given geographical region (Weiser 1970, Fuchigami et al. 1982).

The timing of growth cessation varies with the latitude of origin (Burley 1966, Kuser and Ching 1980, Cannell and Sheppard 1982). A similar latitudinal ecotype differentiation has been reported for bay willow (*Salix pentandra* L.) (Junttila 1980). In North America, strong latitudinal clines for the timing of budset and growth cessation are found in species growing along the Pacific coast, e.g. Sitka spruce (*Picea sitchensis* (Bong.) Carriére) (Burley 1966, Cannell and Sheppard 1982), western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) (Kuser and Ching 1980), and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) (Campbell and Sorensen 1973). There are substantial differences in autumn frost hardiness between interior Douglas-fir (var. *glauca*) populations, with early growth cessation, and coastal Douglas-fir (var. *menziesii*), which has a noticeably later cessation (e.g. Rehfeldt 1977). Lodgepole pine (*Pinus contorta* Dougl. ex Loud.) from the northern part of its range exhibits strong latitudinal and elevational clines for frost hardiness and annual growth ring lignification (Jonsson et al. 1981, Lindgren and Nilsson 1992).

However, the regulation system of growth cessation has still not been fully revealed. Research studies show different results regarding the relationship between growth cessation and the environmental factors. (1) The combined regulation of growth cessation by temperature and photoperiod has been studied extensively (Doorenbos 1953, Vegis 1964, Wareing 1969, Perry 1971, Kramer 1995). In plant shoots, growth cessation is induced by short day, low temperature, or environmental stress (Wareing 1950a,b, Downs

and Borthwick 1956, Aronsson 1975, Levitt 1980, Siminovitch 1981). Temperature interacts with day length to control the timing of growth cessation and budset (Junttila 1980, Downs and Bevington 1981). (2) Fuchigami et al. (1971) have suggested that the induction of growth cessation is the main function of short day in natural cold acclimation. For pine and spruce it is possible to induce both growth cessation and budset by shortening the photoperiod to below a certain critical day length (Dormling et al. 1968). Day length is the triggering factor for growth cessation in forest trees of the boreal zone (Dormling et al. 1968, Dormling 1973, Heide 1974, Jonsson et al. 1981, Hänninen et al. 1990, Repo et al. 1991). (3) After growth cessation, temperature is the main factor responsible for the development of frost hardiness. Owing to the episodic shoot growth patterns of Scots pine and other conifers, the relationships that exist between photoperiod, growth cessation, and cold acclimation are not easily identified. Hence, in Scots pine the prerequisite of short day for inducing cold acclimation does not appear to be the cessation of shoot growth (Smit-Spinks et al. 1985).

The main environmental factor determining the cessation of growth and the cold acclimation of roots is the declining soil temperature at the end of the growing season (Weiser 1970, Smit-Spinks et al. 1985, Rikala and Huurinainen 1990, Ryyppö et al. 1998, Bigras et al. 2001). According to the prevailing view, roots do not have any "real" physiological dormancy, i.e. they grow whenever the soil temperature is high enough.

2.4 Maximum photochemical efficiency of photosystem II (F_v/F_m) during cold acclimation

The maximum photochemical efficiency (F_v/F_m) of the photosystem II (PS II) decreases during photoinhibition (Öquist et al. 1992, Mohammed et al. 1995), and plants subjected to low temperature and high irradiance environments often show significant photoinhibition (Strand and Öquist 1988, Bolhár-Nordenkampf et al. 1989, Örlander 1993).

The winter inhibition of photosynthesis is well established in the case of Scots pine and Norway spruce (Linder and Troeng 1980, Leverenz and Öquist 1987, Lundmark et al. 1988, Ottander and Öquist 1991, Strand and Lundmark 1995) and seems to include both temperature stress *per se* and low-temperature enhanced photoinhibition. It has been shown that F_v/F_m gradually decreases during the autumn as a result of night frosts (Troeng

and Linder 1982, Strand 1995, Lundmark et al. 1998). Placing plants in low temperature environments generally has a negative impact on their photosynthetic electron transport and carbon assimilation (Huner et al. 1993). The proportion of closed PS II reaction centres increases as the rate of photosynthesis decreases at a low temperature. Low temperature also inhibits the rate at which the PS II is repaired (Öquist and Huner 1993).

The findings of previous studies concerning the effects of photoperiod on F_v/F_m are rather conflicting. Greer (1998) has reported that the differences in the fluorescence ratio F_v/F_m between plants in the two treatments of long day and short day were very small. Hence, the differences in F_v/F_m are consistent with the differences in net photosynthesis between plants in the two growth conditions, indicating that the plants in both retained a high photosynthetic efficiency. However, Vogg et al. (1998a,b) has reported that, in the absence of strong frost, the photochemical efficiency was lower under short-day conditions than during a long-day photoperiod. Under the impact of strong frost, the photochemical efficiency was strongly inhibited in both sets of plants.

The efficiency of photosynthetic adjustment as a result of long-term acclimation to a stress also depends on the plant species or variety (Steffen and Palta 1989, Hurry and Huner 1992). In addition to the reaction centres of the two photosystems, the membrane lipids, pigment complexes and cofactor protein complexes (Feierabend and Dehne 1996) all need to be protected against the general consequences of photooxidative stress in the cold and under an excess of light (Wise and Naylor 1987, Feierabend et al. 1992, Sarry et al. 1994). The extent to which all of these changes influence each other and also the overall mechanisms of photosynthetic adjustment is not actually fully understood.

2.5 Sugar concentration during cold acclimation

During cold acclimation many tree species show a marked increase in their soluble sugar concentration, mainly sucrose and its galactosides, raffinose and stachyose (Sauter and van Cleeve 1991). Scots pine carbohydrate dynamics partially depend on inherited properties that are probably related to the phenology of root and shoot growth. Scots pine and other coniferous species are characterized by pronounced seasonal changes in non-structural carbohydrate concentrations in their needles, stems and roots (Oleksyn et al. 2000). Compared with their stems and roots, the needles generally contained the highest

concentration of carbohydrates and exhibited the greatest seasonal change in carbohydrate concentration.

The carbohydrate dynamics are also related to seasonal fluctuations in frost hardiness (Parker 1959). Mechanistic studies have indicated that sugars are directly involved in the hardening process both by reducing the amount of water diffusing from cells when ice crystals are formed in the extracellular spaces at subfreezing temperatures (Hodge and Weir 1993) and by stabilizing cellular structures once ice crystal formation causes cellular dehydration. Sugars are known to lower the freezing point and increase the intracellular osmotic potential. This will reduce the amount of dehydration during extracellular freezing (Levitt 1980).

2.6 Modelling of cold acclimation

Siminovitch et al. (1967) has suggested that total frost hardiness is the result of independent physiological events whose effects are additive. When plants were subjected to more than one environmental factor, the increased hardiness was the sum of the effects of the individual factors involved. Results indicate that low temperature, water stress, and short days initially trigger independent frost-hardening mechanisms (Chen and Li 1978). In the previous models the increasing effect of temperature and photoperiod on frost hardiness was assumed to be additive (Aronsson 1975, Christersson 1978, Chen and Li 1978, Jonsson et al. 1981, Greer and Warrington 1982, Leinonen et al. 1996).

In a model of Repo et al. (1990), it is assumed that there is a discrete stationary level of frost hardiness that is dependent on the prevailing air temperature. In the model itself the relationship between the stationary level of frost hardiness and the daily minimum temperature is assumed to be linear (Repo et al. 1990). The rate of development of the prevailing frost hardiness, i.e. hardening or dehardening, is assumed to be dependent on the difference between the stationary level of frost hardiness and the prevailing frost hardiness (feedback control). Under natural conditions, however, the ability to adjust to a changed environment is not constant throughout the year, and hence the stationary level is probably never reached owing to a delay in the changes in frost hardiness resulting from the fluctuating environmental conditions. In the model produced by Leinonen et al. (1996), the effect of the photoperiod was also addressed. The response of the stationary level of

frost hardiness to temperature and photoperiod was assumed to be linear in a piece-wise manner and additive (Leinonen et al. 1996).

Although a great deal of information already exists on the effects of short photoperiods and low temperatures on frost hardening (Christersson 1978, Smit-Spinks et al. 1985, Bigras and D'Aoust 1993), the information remains largely qualitative in nature. No attempt has been made to identify the underlying processes that regulate the development of frost hardiness in different organs. Knowledge of the quantitative relationships between the frost hardening process and the major environmental factors involved is also needed for developing models that can simulate seasonal changes in frost hardiness (Kobayashi et al. 1983, Anisko et al. 1994, Timmis et al. 1994, Leinonen 1996).

2.7 Frost hardiness assessment methods by controlled freezing tests

2.7.1 Visual scoring of damage

When exposure to freezing temperatures is applied, the visual scoring is a simple, straightforward method for approximating the effects of a similar exposure under natural conditions. The test results are often evaluated by means of visual estimation of the percentage of browning of damaged tissue after an incubation period; hence references to the 'browning test' (Glerum 1985).

2.7.2 Electrical impedance spectroscopy

Electrical impedance spectroscopy (EIS) is a method used for studying the structure of organic and inorganic materials (Ackmann and Seitz 1984, Foster and Schwan 1989, Macdonald 1992, Repo and Zhang 1993). In plant physiology, impedance analysis has been used, for example, to assess heat, freeze, salt and electric stress (Zhang and Willison 1992, Zhang et al. 1993, Repo et al. 1994, Inaba et al. 1995, Mancuso and Rinaldelli 1996) as well as fruit quality and ripening (Cox et al. 1993, Harker and Maindonald 1994, Varlan 1996). Information of fundamental value concerned with the physiology of organisms can be obtained by measuring the impedance spectra of tissues and organs (Cole 1968, Ackmann and Seitz 1984).

When alternation current (AC) is applied to a piece of plant tissue, the proportion of current going through the extracellular space and that going through the intracellular compartment vary with both the AC frequency and the tissue properties (Wilner and Brach 1979, Glerum 1980). Thus, information about extra- and intracellular fluids can be obtained by working with a sufficiently wide frequency range. When a tissue is represented by an electrical circuit, the tissue features (structural as well as physiological) can be quantified by means of an electrical circuit analysis (Repo et al. 1994). This kind of analysis of a system, based on the impedance data measured at various AC frequencies, is known as EIS (Macdonald 1987). With a proper equivalent model it is possible to study the effects of different stress factors on the tissue structures according to the changes in the parameters of the model (Zhang and Willison 1992, Zhang et al. 1993, Repo et al. 1994). When the cells are undamaged, they are able to retain a high intracellular ion concentration with respect to the extracellular space. If the cell membranes are injured by frost they lose their ability to maintain a high intracellular concentration (Palta and Li 1978). As a result, the concentration gradients between the intra- and extracellular spaces disappear. The damage can be regarded as a reduction in the low-frequency impedance (Repo 1988).

One advantage of this method is that it is easier to make electrical measurements on a sample than to submit it to time-consuming chemical testing. Given the recent advances in measurement and analysis, electrical impedance technology has become a reliable and sensitive method of detecting changes in plant cell structures resulting from freezing injury. In determining frost hardiness, the extracellular resistance (r_e) is the most appropriate parameter to measure after a controlled freezing test (Repo et al. 1994, 1997, Ryyppö et al. 1998). The reduction in r_e coincides with changes in the plasma membrane ATPase activity and also in relative electrolyte leakage, and is thus the result of damage in the cell membranes, which lose their capacity to maintain the electrochemical gradient between the intra- and extracellular space. The determination of frost hardiness can be conducted by estimating the inflection point of a logistic sigmoid function of the extent of damage vs. freezing temperature by non-linear regression (Repo and Lappi 1989).

2.7.3 Electrolyte leakage

For decades, in order to estimate stress damage measurement has been made of the leakage of electrolytes from plant tissues (Dexter et al. 1932). Electrolyte leakage was used in the 1950s to compare the level of injury caused to the roots and shoots of woody plants by frost (Wilner 1959), and it has since been used widely to assess frost hardiness. The principle of the electrolyte leakage method is based on changes in the cell membrane properties controlling the electrochemical gradient (Steponkus 1984, Pukacki and Pukacka 1987) that occur during or after exposure to injurious low temperatures. The movement of cell contents to and from cells is mainly controlled by the structural proteins present at points along the lipid bilayer of the cell membrane. When healthy tissue is put in water that is almost free of ions, there is a slight leakage of the cell contents, including ions, into the surrounding water which can be detected with a conductivity meter. If the cell membrane is ruptured or the transmembrane protein pumps impaired, the cell contents leak at a higher rate. The electrolyte leakage rate is, therefore, a measure of damage to the cell membranes (McKay 1992).

2.8 Frost hardiness prediction on the basis of non-frost-exposed samples

The methods generally used for predicting the frost hardiness of plants require fairly expensive equipment for conducting freezing tests and may be too laborious for large-scale monitoring. Furthermore, an efficient study of the biochemical and physiological changes occurring during hardening requires an easy and rapid, non-destructive method of assessing the frost hardiness of the sample. Hence, there is a great challenge to develop a rapid method for predicting frost hardiness that might not necessitate controlled freezing testing (Repo et al. 1997).

2.8.1 Electrical impedance spectroscopy

Several equivalent circuit parameters vary seasonally and in relation to the developmental changes in plant tissues associated with cold acclimation. Changes in cell constituents with acclimation point towards the conclusion that there may also be changes in equivalent

circuit parameters that could be used for assessing the frost hardiness of plants without controlled freezing tests. Some of the measured electrical impedance parameters have revealed a seasonal pattern which coincides with the changes in frost hardiness (Stout 1988a,b, Repo et al. 1995). In the stems of Scots pine, the intracellular resistance increased with increasing frost hardiness (Repo et al. 1995, 1997). Impedance analysis is one of the most promising methods for assessing frost hardiness without employing a controlled freezing test (Burr et al. 2001).

2.8.2 Maximum photochemical efficiency of photosystem II (F_v/F_m)

Chlorophyll fluorescence is a sensitive and early indicator of damage in the process of photosynthesis and in the plant in general, resulting from stresses such as freezing, chilling, and drought (Ögren and Öquist 1985, Schreiber and Bilger 1987, Hetherington and Öquist 1988, Strand and Öquist 1988, Adams et al. 1989, Gamon and Pearcy 1989, Krause and Weis 1991, Epron et al. 1992, Groom and Baker 1992, Larcher 1995, Werner and Correia 1996, de Mattos et al. 1997, Maxwell and Johnson 2000). As pointed out by Ball et al. (1995), the functioning of photosystem II (PS II) is one of the most sensitive indicators of environmental stress in plants. The photochemical efficiency of PS II, which is frequently determined as an F_v/F_m ratio, is derived from chlorophyll a fluorescence measurements. The parameter F_v is equal to the maximum fluorescence (F_m) minus the ground level of fluorescence (F_o), where F_m is the maximum fluorescence at saturating light pulse when all the PS II centres become fully reduced, and F_o is fluorescence in very low light when all the PS II centres are fully oxidised (Lamontagne et al. 2000). The ratio of F_v/F_m can serve as an excellent quantitative measure of photoinhibition (Björkman and Demmig 1987, Krause and Weis 1991).

The principle underlying chlorophyll fluorescence analysis is relatively straightforward (Maxwell and Johnson 2000). Light energy absorbed by chlorophyll molecules in a leaf can experience one of three fates: it can be used to drive photosynthesis (photochemistry), excess energy can be dissipated as heat, or it can be re-emitted as light—chlorophyll fluorescence. These three processes occur in competition, so that any increase in the efficiency of one will result in a reduction in the yield of the other two. Hence, by measuring the yield of chlorophyll fluorescence, information about changes in the efficiency of

photochemistry and heat dissipation can be gained. During the saturating light pulse, the fluorescence yield reaches a value equivalent to that which would be attained in the absence of any photochemical quenching, the maximum fluorescence, F_m. Comparison of this value with the fluorescence yield at a very low photon flux density (F_o) provides information about the efficiency of the photochemical quenching and, by extension, about the performance of PS II (Maxwell and Johnson 2000).

2.8.3 Dry matter content

The relation between the dry matter content and frost hardiness offers an explanation for the observation that during the autumn the dry-to-fresh weight ratios of shoots and stems and their frost hardiness increase in parallel (Pellett and White 1969). The dry matter content of woody plant shoots increases with cold acclimation (Junttila and Kaurin 1990, Toivonen et al. 1991, Repo et al. 1997). As a result of its simplicity, the method used appears to have practical applicability, e.g., in nurseries. The predictive power of the dry matter content for assessing the frost hardiness is variable, however. In previous studies, the dry matter content of plant tissues started to increase before the frost hardening had started. The increase in the dry matter content proceeded faster than the frost hardiness itself increased during the first stage of cold acclimation, and the change in the dry matter content also ended earlier than the frost hardening ceased (Sutinen 1992, Repo et al. 1997).

2.8.4 Sugar concentration

The development of frost hardiness is strongly correlated with the change in the carbohydrate content (Sakai and Yoshida 1968, Aronsson et al. 1976, Chen and Li 1980, O'Neill 1983, Ögren et al. 1997, Greer et al. 2000). Many high correlations have been found between increases in frost hardiness and increases in chemical substances such as sugars, proteins, amino acids, lipids and nucleic acids. These increases in substances result in a general protoplasmic augmentation during hardening (Siminovitch et al. 1967, Pomeroy et al 1970). It has also been found that the rate of hardening increases with increasing concentrations of hexose sugar concentrations in seedling shoots (Greer et al.

2000). The maximum concentrations of carbohydrates and maximum frost hardiness were not, however, observed simultaneously in the canes and buds of three red raspberry cultivars (*Rubus idaeus* L. 'Maurin Makea', 'Ottawa', and 'Muskoka') (Palonen 1999).

3 MATERIALS AND METHODS

The materials and methods are briefly described here since more detailed information is available in the original papers I-IV.

3.1 Plant materials, growing conditions and acclimation procedures

Scots pine saplings (I-III) and seedlings (IV) were used in the studies. In Studies I-III, 8-year-old saplings with 6 different origins grown in central Finland in the Research Nursery at the Suonenjoki Research Station of the Finnish Forest Research Institute were used. These origins ranged from Estonia to northern Finland, thus forming a latitudinal gradient of ca. 10°N. Sixteen sample saplings from each origin were chosen according to the mean height of the origin. Samples were taken at 2-3 week intervals between 3 August and 30 November 1998 (I-III). Current shoots from lateral branches were sampled for the assessment of FH by means of controlled freezing tests (I-III), for impedance analysis of stems (II) and needles (III), and for determination of the DM content of the stems and needles (II-III).

For Study IV, second-year seedlings of central Finnish origin were first grown at the Suonenjoki Research Station for the first year during 1999 and then in the second year, between 2 May and 2 July 2000. The seedlings were raised using the normal nursery routines at the Research Nursery. On 3 July 2000 the seedlings were then transferred to the Faculty of Forestry at the University of Joensuu so that the experiment could be conducted in the growth chambers located there. After a growing period of one week in these chambers under the same controlled environmental conditions, the second-year seedlings were subjected to different photoperiods and temperatures, i.e. a short photoperiod (7/17h; day/night) at a high temperature (15°C) (SDHT), a short photoperiod

(7/17h; day/night) at a low temperature (2°C) (SDLT), and a long photoperiod (16/8h; day/night) at a low temperature (2°C) (LDLT), with two replicates for each treatment.

3.2 Measurements of shoot growth

In Study I, the elongation of the current-year shoots, the diameter growth of the current-year shoot, and the elongation of the current-year needles were each monitored weekly from 11 May to the end of August 1998. Growth cessation was determined as the date when the mean shoot and needle elongation and the mean diameter growth reached 90% of their respective final levels.

In Study IV, the stem diameter at 1cm above the peat surface, the shoot elongation (height growth), and the needle elongation were measured weekly between 11 May and 4 August 2000. At the nursery a total of 50 sample seedlings were chosen randomly from all of the seedlings growing in trays, while in the growth chambers 16 sample seedlings in a single tray placed in each chamber were selected according to the mean height of the seedlings.

3.3 Controlled freezing tests

For the assessment of FH, the separate organs of the stems (I, II and IV), needles (I-IV), buds (IV) and roots (IV) of the Scots pine saplings and seedlings were exposed to frost in air-cooled freezing chambers. The rate of cooling and warming was 5°C h^{-1}, and the duration of the target minimum temperature was 4 h. In the course of each exposure period, 6-7 temperatures and controls at either +3°C (I-III) or +5°C (IV) were applied. The exposure temperature range used in the freezing tests was between 0°C and –130°C in Studies I-III and between 0°C and–80°C in Study IV, depending on the origin, organ, treatment and developmental stage of the plants.

3.4 Determination of frost hardiness

After the freezing test, samples were used for measurements of the FH by means of electrical impedance (I, II and IV), electrolyte leakage (I-IV), and the visual scoring of damage (I).

The extracellular resistance obtained by means of the electrical impedance method (Repo 1994, Repo et al. 1994) was measured in the stems of current shoots of Scots pine saplings and seedlings (I, II and IV). The impedance spectra were modelled on the basis of the double-DCE (distributed circuit element) model (Repo et al. 1994). The stems were cut into 15 mm sections for the performance of the measurements. First, a temperature response curve was produced, and then the frost hardiness was obtained as an inflection point by means of curve fitting.

The electrolyte leakage (Flint et al. 1967, Burr et al. 1990, Sutinen et al. 1992) was measured in the needles (I-IV), buds (IV) and roots (IV) of the Scots pine saplings and seedlings. The needles and roots were cut into 10 mm sections for the performance of the measurements.

Visual damage was scored for the needles of the Scots pine saplings (I). The FH assessment was based on estimation of the browned needle area. The damage was scored one week after the freezing test had been conducted. The FH was obtained by interpolation and defined as the temperature at which 50% of the needles were affected (LT_{50}).

3.5 Measurements of the non-frost-exposed organs

For the non-frost-exposed organs, i.e. for the samples that were not exposed artificially to frost in the controlled freezing tests, the EIS of the stems (II, IV) and needles (III), the DM content of the stems (II, IV) and needles (III, IV), and the F_v/F_m of the needles (IV) were assessed immediately after sampling. The sugar concentrations in the stems, needles and roots were assessed subsequent to the DM content measurements by using the same samples (IV).

The impedance spectra of the stems were modelled on the basis of an equivalent circuit with two distributed circuit elements (DCE) in series with a resistor (double-DCE model) (Repo et al. 1994). The impedance spectra of the needles were modelled on the

basis of an equivalent circuit Model-A (an equivalent circuit which takes account of the presence of air spaces within the needles; the letter A stands for air space) (Zhang et al. 1995). For both stems and needles, 15 mm sections were cut for measurements. The resistance parameters were normalised with respect to the cross-sectional area and the length of the stem and needle samples in order to obtain the specific resistances.

After fresh weight measurements, the samples were oven-dried at 80°C for 48 h in Studies II and III, and at 60°C for 48 h in Study IV before weighing for their dry weight. The DM content was calculated as the percentage of the dry weight in relation to the fresh weight. For stems each of the 16 samples were measured, whereas in the case of the needles and roots all of the pooled 16 samples were measured on each measuring occasion.

The chlorophyll fluorescence of the dark-adapted (20 min in Study IV) needles was measured in the laboratory at room temperature of 24°C. The maximum photochemical efficiency of PS II was determined as the ratio of variable to maximum fluorescence (F_v/F_m), where F_v was calculated from F_m-F_o (F_o is ground fluorescence).

Concentrations of soluble sugars were analysed in the stems, needles and roots (IV), as described by Hansen and Møller (1975). Shortly after being dried at 60°C for 48 h, each of the samples per treatment was ground to a powder. The total soluble sugars in the pooled supernatant were determined colorimetrically at 630 nm with anthrone, using D-glucose as a standard.

3.6 Statistical analyses

Statistical analyses were carried out using Microsoft Excel and SPSS (SPSS 8.0 for Windows, SPSS Inc.). The data was presented both as means and as standard error of the means. In Studies I-III, the 6 provenances were divided into 3 groups on the basis of the latitude of origin, each group being represented by two provenances. One-way Analysis of Variance (ANOVA) was used to calculate the data related to the cessation of growth and FH at the different times of the measurements (I), and also to the electrical impedance parameters, DM content, F_v/F_m and sugar concentrations and FH at the different times of the measurements (IV). The Univariate Analysis of Variance (SPSS 8.0 for Window, SPSS Inc.) was used to analyse the significance of the mean difference in the equivalent circuit

parameters between the groups (III). The paired samples *t*-test was used in order to make a multiple comparison of the FH at the different times of the measurements (I and II), and the equivalent circuit EIS parameters and DM content as they existed between the groups (II). Linear regression analysis was applied in order to analyse the relationship between the traits (I and II), and exponential regression analysis was used for calculating the relationship of the FH of organs to the DM content of the stems (II). A quadratic regression model was used to compare the different traits (III). The differences between the LT_{50} values in the different phases of hardening were considered to be significant if the values of Wald's χ^2-test 95% confidence intervals did not overlap (I and IV). For the evaluation of the reliability and accuracy of the regression models, the coefficient of determination (Microsoft Excel: II and III; SPSS Inc: IV), the confidence intervals and the residuals (SPSS Inc.) (II) were all examined.

4 RESULTS

4.1 Growth cessation in Scots pine of different origins and organs

Growth ceased first in the northern origins and last in the southern ones (I). The shoot elongation, diameter growth of the stem, and needle elongation ceased in the northernmost origin 10, 16 and 16 days earlier than in the southernmost origin, respectively (I). When compared for the different organs, shoot elongation stopped earliest, needle elongation next, and stem diameter growth last (I, IV).

When a comparison was made between growth cessation and the timing of the initial hardening, the northernmost origin reached a FH of –10°C and –15°C 21 and 10 days earlier than the southernmost origin, respectively, which accords with their growth cessation (I). A linear correlation was found between growth cessation (shoot elongation, stem diameter growth and needle elongation) and the initiation of frost hardening to –10°C and –15°C in Scots pine origins (I). The difference in temperature sum at the time of the cessation of shoot elongation was 110 degree days (d.d.) between the northern and southern origins (I).

4.2 Frost hardiness in Scots pine of different origins and organs

The FH of the stems was between –5°C and –6.5°C for Scots pine saplings in the different origins (I) and –6°C for Scots pine seedlings from a central Finnish origin (IV) when no hardening occurred. Under field conditions in August, the FH for each of the stems revealed no differences between their origins (I). Frost hardening was initiated in September, first in the northern origins and last in the southern ones, and the stems were also found to harden faster in the northern origins than those in the southern ones (I). From the beginning of September until early October the differences in FH between the origins was significant (I). At the end of November, the FH in stems of different origins did not differ (I). In the growth chambers under controlled environmental conditions, the FH of the LDLT and SDLT treatments was higher than in the SDHT treatment applied in the beginning stage (IV), while at the end of the treatments no differences were revealed between the various treatments (IV).

The FH of the needles was –5°C to –8°C for the Scots pine saplings of different origins (I) and –8°C for the Scots pine seedlings of central Finnish origin (IV) when no hardening occurred. In Study I, the FH of the needles assessed by the electrolyte leakage method showed no differences between the various origins in August except that the needles from the northernmost origin were hardier than those from the other origins (I). Frost hardening was initiated at the beginning of September. The needles from the northern origins hardened earlier and faster than those from the southern origins (I). At the end of November, the FH in needles of different origins had the same levels, ranging from –80°C to –90°C (I). In Study IV, after the commencement of the treatments the needles in the LDLT treatment were significantly less frost hardy than in the SDHT and SDLT treatments between days 36 and 50 of the hardening. At the end of the treatments, however, no differences were found in the FH of the needles in the various treatments (IV).

The FH of the buds was initially about –8°C in the Scots pine seedlings of central Finnish origin (IV). In the growth chambers, after the commencement of the treatments the buds in the SDLT treatments were significantly frost hardier than those in the SDHT and LDLT treatments during the initial stages of the treatments (IV). At the end of the treatments, no difference was discovered in the FH between the LDLT and SDLT treatments (IV).

Initially, the FH of the roots was approximately –6°C for the Scots pine seedlings of central Finnish origin (IV). During the process of the controlled environmental condition, the roots in the LDLT and SDLT treatments became significantly hardier than those in the SDHT treatment, a difference which continued to the end of the treatments (IV).

When compared with the organs from Scots pine saplings growing under field conditions, the FH for both stems and needles was found to be approximately the same from early August until the beginning of September (I). Thereafter, the FH in the needles increased more quickly than that in the stems, and the needles became much hardier than the stems. A clear linear relationship between the FH of the stems and needles was in existence throughout the experiment, from early-August to late-November (I).

In the case of the Scots pine seedlings of central Finnish origin, the initial FH of the stems and roots was lower than that of the needles and buds, i.e. –6°C and –8°C, respectively (IV). At the end of the treatments, the FH followed the sequence: needles > buds > stems > roots (IV).

4.3 Changes in the properties of non-frost-exposed organs and their relation to frost hardiness

4.3.1 Electrical impedance spectroscopy

The shape of the impedance spectra of the non-frost-exposed stems and needles in Scots pine saplings changed during the study period, which lasted from early-August to late-November 1998 (II, III). Two arcs dominated in the stems especially in early August, and with frost hardening the proportion of the low and high frequency arcs altered as the high frequency arc became more dominant (II). In the case of the needles, in early August the spectra were characterized by two strongly overlapping arcs, and later only one arc was present (III).

Both for Scots pine saplings with different origins and for seedlings undergoing different environmental treatments, the trend in the equivalent circuit parameters of the stems was similar (II, IV). The extracellular resistance r_e, intracellular resistance r_i and relaxation time τ_1 increased, while initially the resistance r_2 decreased (II, IV). For most of the time, the differences between the origin groups were significant in Study II, and the significant

differences between the treatments in Study IV were also found in r_e, r_2 and Ψ_1 (distribution coefficient of τ_1).

The changes in the equivalent circuit parameters of the needles employing the Model-A were divided into two phases (III). In the first phase, from August to about mid-September, the r_e decreased and the β parameter (a factor in Model-A controlling spectrum skewness and impedance locus centre depression) first declined and then increased. In contrast, the r_i and membrane time constant τ_m first increased and then fell (III). In the second phase, the r_e started to increase in mid-September and the r_i increased at the beginning of October (III). The parameters in many occasions differed significantly between the various origins (III).

The relaxation time τ_1 and the intracellular resistance r_i of the stems in Scots pine saplings had the highest correlation with the FH of the stems and needles (II). The linear regression model described the relation between the FH and τ_1 well for the pooled data (II). At selected times in the hardening phase the correlation between the FH and the τ_1 was higher and the residuals between the predicted and measured FH lower than for the pooled data (II). At its highest, the coefficient of determination was 0.95 between the FH of the needles and the τ_1 of the stems on 21 September. At that stage the residuals were within $\pm$2.0°C (II).

No correlation was found between the equivalent parameters of the non-frost-exposed needles and the FH during the whole experimental period from early-August to late-November 1998 (III). Between late-August and early-September significant differences occurred between the origin groups in their τ_m, and the τ_m declined concomitantly with an increase in the FH during that period. From early-October to late-November the intracellular resistance r_i differed significantly from one provenance group to another, and the r_i increased concomitantly with an increase in the FH during that period (III).

4.3.2 Maximum photochemical efficiency of photosystem II (F_v/F_m)

In Study IV, the F_v/F_m differed significantly between treatments. With the low temperature treatment, i.e. LDLT and SDLT, the F_v/F_m clearly decreased while in the case of SDHT it scarcely changed during the treatment (IV). When the data from all of the treatments was

pooled, in the case of the Scots pine seedlings there was no correlation between the F_v/F_m of the needles and their FH (IV: data not shown).

4.3.3 Dry matter content

The DM content of the stems (II, IV), needles (III, IV) and roots (IV) increased during cold acclimation. The increases in the DM content occurred earlier than the increases in the FH. The DM content of the stems in the northern and intermediate origin groups was almost always significantly higher than the DM content for the southern origin group in Study II. In contrast, the DM content of the needles (III) displayed had no significant differences between the origin groups except in the case of the intermediate and northern groups in October. In Study IV, the DM content of the needles in the LDLT and SDLT yielded higher values than the SDHT treatment, while the DM content of the roots in the SDLT was higher than for the other treatments by the end of their application.

The relation between FH and the DM content was non-linear in Scots pine saplings (II, III). The DM content increased at first without hardening, and when the frost hardening was initiated the DM content reached a level of between 32% and 35% for the stems and between 25% and 34% for the needles of the Scots pine saplings (II). The predictive power of the DM content was variable; an increase in the DM at low values had no effect on the FH (II).

4.3.4 Sugar concentration

The total soluble sugar concentration of the stems, needles and roots increased in the low temperature treatments (LDLT and SDLT). In contrast, in the SDHT treatment the changes were few and remained at the lowest level in the case of all of the organs (IV). There were significant differences between the organs in terms of their sugar concentrations, especially between the roots and the other two organs (IV).

The sugar concentration in the various organs followed the sequence needles > stems > roots, which matched the levels of their FH (IV).

4.4 Modelling of cold acclimation

The FH of the roots became stationary when the environmental conditions remained constant (IV). In the case of the stems and needles, the estimation of the stationary frost hardiness proved that the asymptotical stationary level of the FH was approached at the end of a 78-day treatment period in Study IV.

In the initial phase of the environmental treatments in Study IV, the frost hardening of the stems was driven mainly by low temperature, whereas the frost hardening of the needles was predominantly affected by a short photoperiod. The frost hardening of the roots was driven principally by low temperature throughout the treatments. When the third measurement was performed (day 36), the FH of the buds was the additive increase in SDLT treatment. When the stationary stage or the asymptotically approaching stationary stage was reached, none of the organs followed the principle that the FH of an organ is an additive property of photoperiod and temperature.

5 DISCUSSION

5.1 Growth cessation of different origins and the relationship with frost hardiness

Growth cessation followed a north-to-south latitudinal trend (I). This agreed with previous studies conducted by Oleksyn et al. (1992) in connection with different European Scots pine populations and also with several other tree species, e.g. in *Picea abies* (Heide 1974, Junttila and Skaret 1990), *P. glauca* (Junttila and Skaret 1990), *Acer saccharum* (Kriebel 1957) and *Salix pentandra* (Junttila and Kaurin 1985). Shoot elongation ceased long before the cessation of needle elongation and diameter growth, as was found in previous studies (Huikari and Paarlahti 1967, Raulo and Leikola 1974, Koski and Sievänen 1985, Ögren 1999a,b).

Growth cessation marks the onset of frost hardening. A linear correlation existed between growth cessation, i.e. shoot elongation, needle elongation and diameter growth, and the initiation of frost hardening in Scots pine origins (I). This coincided with the results obtained for the relationship between bud set and the initiation of frost hardening in the

stems and needles of first-year Scots pine seedlings at the population level (Hurme et al. 1997), and for *Picea abies* (Johnsen and Apeland 1988, Westin et al. 1995), *Pseudotsuga menziesii* (Campbell and Sorensen 1973, Rehfeldt 1979, 1988, 1989, Loopstra and Adams 1989), *Picea mariana* (Mill.) BSP (Sulzer et al. 1993) and *Pinus radiata* (Greer et al. 2000). In view of the close coordination between growth and hardening cycles, there is a risk that breeding for increased FH will lead to earlier growth cessation, and hence to a loss of growth potential (Ögren 1999a,b). This was deduced from the genetic differences in the prehardening value of the FH and in the timing and rate of frost hardening, and hence there seems to be considerable scope for improving frost hardening through breeding and selection.

The growth cessation of shoot elongation in Scots pine saplings was related to the temperature sum (in d.d.), in accordance with previous studies (Wright 1976, Koski and Sievänen 1985). However, the southern origins required higher temperature sums for their growth cessation than the northern ones did, in contrast to the results produced by Koski and Sievänen (1985). Accordingly, it may be the case that either the photoperiod or the joint effect of the photoperiod and temperature has some impact on growth cessation in Scots pine saplings, or origins have different thresholds of temperature sum for growth cessation. The first-year seedlings of the Scots pine, seedlings of *Picea abies* (L.) Karst. and *Betula pendula* Roth. display the combined effect of temperature sum and photoperiod in the growth cessation of shoot elongation (Koski and Selkäinaho 1982, Koski and Sievänen 1985, Oleksyn et al. 1992). However, the influencing factors are still under debate. Partanen and Beuker (1999) have suggested that Scots pine seedlings carry genetic information which governs the regulation system of growth cessation. They also suggest that the responses to temperature and photoperiod have separate genetic controls. In many woody species originating in the temperate zone, growth cessation and the development of hardiness are initiated by photoperiod (Nissilä and Fuchigami 1978, Kobayashi et al. 1983, Sakai and Larcher 1987). The exact roles of the accumulation of temperature sum and decreasing day length in governing bud formation are also still under debate (Koski and Sievänen 1985, Hänninen et al. 1990). Further experimental studies are needed so that knowledge about the joint effects of temperature sum and photoperiod on growth cessation and frost hardening can be improved.

5.2 Frost hardiness by controlled freezing tests

The FH of Scots pine saplings in both their stems and their needles from various different origins ranged between –5°C and –8°C, while that of second-year Scots pine seedlings in terms of needles and buds of central Finnish origin was –8°C, a little higher than that of stems and roots (–6°C) in cases where no hardening was initiated (I, IV). In previous studies, at the end of the growing season, the FH of stems and needles varied between –5°C and –8.5°C for first-year seedlings (Hurme et al. 1997), between –4°C and –5°C for second-year seedlings (Rikala and Repo 1987, Repo et al. 1994, Ryyppö et al. 1997), and from –3°C to –5°C in 15- to 20-year-old specimens of Scots pine and Norway spruce at the stage when shoot elongation was approaching cessation (Repo 1992).

Frost hardening was initiated firstly in the northern origins, next in intermediate origins, and finally in southern origins (I). This agrees with previous studies made of first-year (Dormling et al. 1977, Nilsson and Eriksson 1986, Toivonen et al. 1991, Aho 1994, Sundblad and Andersson 1995, Hurme et al. 1997) and older seedlings and trees of Scots pine (Lindgren and Nilsson 1992, Dormling 1993, Andersson 1994, Nilsson and Walfridsson 1995, Beuker et al. 1998), and also of other tree species, such as *Pinus contorta* (Jonsson et al. 1981), *Picea glauca* (Simpson 1994), *Pseudotsuga menziesii* var. *glauca* (Rehfeldt 1979), *Chamaecyparis nootkatensis* (D. Don.) Spach (Hawkins et al. 1994), and *Alnus* species (Tremblay and Lalonde 1987). In both Scots pine and Norway spruce, the northern origins started to harden earlier than the southern ones even though the trees had been growing for over 60 years under the same photoperiod and temperature conditions (Beuker et al. 1998). This indicates (1) strong genetic control of the FH, and (2) that selection, e.g., through repeated thinning (Beuker 1994), has not caused differences between the origins to disappear. With fully hardened samples the effect of latitudinal origin became insignificant, and all of the origins studied in Study I were hardy enough to tolerate the harsh winter conditions. This means that, in the case of Scots pine, the maximum hardening potential would not be a limiting factor for south-to-north origin transfers, but rather the timing of frost hardening may very well play a significant role (Repo et al. 2000b).

After the initiation of hardening, the FH of the needles of Scots pine saplings of different origins increased more quickly than that of the stems, while the needles were much hardier than the stems (I). Under the artificial conditions created in Study IV, the FH was highest in

the needles, followed by the buds and stems, with the roots proving to be the least hardy. These results agree with earlier results produced with seedlings of Scots pine and white spruce (*Picea glauca* (Moench) Voss), where the stems hardened more slowly than the needles under artificial conditions, and the frost hardening was deeper in the needles than in the stems (Bigras and D'Aoust 1992, Hurme et al. 1997, Ryyppö et al. 1998). This was also the case with silver fir (*Abies alba* Mill.), where the needles were hardier than the buds (Sakai and Larcher 1987, Beuker et al. 1998). This means that the responses of buds, stems and needles to the changing environmental conditions differ in the course of the autumn. The results could be explained, firstly, by positing the joint effect of photoperiod and temperature (Aronsson 1975), and, secondly, in terms of diameter growth, which continues longer than bud set and needle elongation and may delay the frost hardening of stems compared to the buds and needles (Repo et al. 2000b, Repo et al. 2001). Regardless of the acclimation treatment, roots were found to be less hardy than shoots. In nature, it must be emphasised that shoots are exposed to temperature extremes in the air from which the roots are insulated by the soil (Smit-Spinks et al. 1985, Bigras and D'Aoust 1992, Coleman et al. 1992, Colombo et al. 1995, Sutinen et al. 1996, Ryyppö et al. 1998). Consequently, in comparison with shoots, the FH of roots may be limited by environmental, physiological, biochemical, and morphological factors.

5.3 Impedance spectra of the non-frost-exposed stems and needles

Both in Scots pine saplings of different origins and in Scots pine seedlings under controlled conditions, the relaxation time τ_1 and the intracellular resistance r_i of the stems had highest correlations with the FH of the stems (II; in Study IV the data was not shown) and needles (II). In mathematical terms, the τ_1 is obtained from the apex of the high frequency arc of the impedance spectrum. Results showed that the increase in τ_1 is caused by the change in the ion mobility in a cellular compartment represented by r_1 (Repo et al. 2000a). The relaxation time τ_1 and the resistance r_1 increased with any increase in the DM content. Thus, the water content may partially explain the behaviour of τ_1 and r_1. An exchange or, more specifically, a redistribution of water between the symplast and apoplast may partially explain the reduction in the τ_1 in the case of freezing tests too (Repo et al. 1994). The net water content of the tissues does not change there, but there is a drift of water towards apoplastic ice by

means of exosmosis. This, together with damage in cell membranes and consequent ion leakage from the symplast to the apoplast (Palta and Weiss 1993, Ryyppö et al. 1998), will lead to a decrease in τ_1. This may, in turn, contribute to a decrease in the r_1 as a result of an increase in the charge-carrying ion content inside or outside the cell (Repo et al. 2000b).

Although no correlation was discovered between the equivalent parameters of the non-frost-exposed needles and the FH, within a particular time period the τ_m (from late-August to early-September) decreased and the r_i (from early-October to late-November) increased concomitantly with an increase in the FH (III). In August and mid-September the most substantial change occurred in the cell membrane time constant τ_m, which initially increased and then decreased. It may be assumed that the needles had reached their maturation phase before the end of August. According to the ultrastructure, in the course of a substantial change phase the needles were seen to satisfy the prerequisites for cold acclimation. Having reached that point, the needles were fully matured and were ready to undergo freezing-stress. In the phase connected with pre-hardening and the initiation of hardening itself, i.e., from the end of August to mid-September, the τ_m decreased at the moment when the initiation of frost hardening made its very first appearance (III). A linear relationship between the cessation of needle elongation and the time to reach τ_m 10ms (R^2=0.58) was found in Scots pines of different origins. In Study I, which is concerned with growth cessation and frost hardening in Scots pines, the cessation of needle elongation was also shown to bear a linear relationship with the initiation of frost hardening down to –15°C (Repo et al. 2000b). These results indicate that during the initiation phase of frost hardening the membrane time constant τ_m changes according to the cessation of needle elongation and accordingly would correlate with the FH. Since $\tau_m=R_3 \bullet C_m$, the big change in the τ_m during the pre-hardening phase is affected by the specific membrane resistance R_3, while the specific membrane capacitance C_m is assumed to be a constant of 1 $\mu F/cm^2$ (Zhang et al. 1995).

The intracellular resistance r_i in the stems increased with the increase in hardiness in the autumn (II), which was found in Scots pine (Repo et al. 1995), alfalfa (*Medicago stativa* L.), birdsfoot trefoil (*Lotus corniculatus* L.) (Stout 1988a,b) and willow (*Salix viminalis*) (Repo et al. 1997). During the phase connected with the substantial increase in the FH, i.e., from early-October to late-November, the intracellular resistance r_i in the needles increased

with the FH (III). A linear relationship existed between the r_i and the FH with an R^2 of 0.51 (III). Electrical resistance is inversely related to the concentration of electrolytes and their mobilities. In the initial stages of hardening, sugars accumulate in cells. As has been previously established, not only are sugars directly involved in the hardening process (Hodge and Weir 1993, Ögren 1999a,b) but they are low mobility electrolytes. An increase in the sugar concentration without a commensurate increase in high-mobility electrolytes would result in a net increase in cytoplasmic resistance (Colombo and Blumwald 1992). In addition, cell compartmentalization increases during frost hardening, and this effect, combined with an increase in viscosity resulting from the accumulation of sugars and other cellular constituents (Stout 1988a,b), may result in a reduction in ion movement and an increase in intracellular resistance. This may help to explain the increase in the r_i of the needles between early-October and the end of November in Study III. During this particular phase the DM content changed only slightly.

The resistance r_2 decreased during the growing season well before any indications of an increase in hardiness appeared (II). The changes in r_2 are probably connected with the initiation of cellular differentiation and lignification in the stem. In Study IV, the EIS parameters r_e, r_2 and Ψ_1 showed that some treatment effects in stems were evident. Those parameters clearly differed in the SDHT treatment from the other two treatments.

The impedance parameters can provide information about structural changes in the cell during the annual cycle of the plant. Further studies are necessary to determine the sensitivity of this technique to change in membrane components and to obtain a more comprehensive biological interpretation of impedance parameters. Further research is also needed into different kinds of plant material and with varying growing regimes concerning the relationship between relaxation time as well as intracellular resistance and FH and the biological interpretation of the equivalent circuit parameters of the distributed models.

5.4 Maximum photochemical efficiency of photosystem II (F_v/F_m) of the non-frost-exposed needles

During cold acclimation, the F_v/F_m decreased in an environmental treatment trial of Scots pine seedlings (IV). The decreases in F_v/F_m were mainly affected by low temperatures. The F_v/F_m of needles in the SDHT treatment remained almost constant (0.75-0.85) during Study

(IV) and they corresponded to the values of unstressed plants (Björkman and Demmig 1987, Bolhár-Nordenkampf et al. 1989, Krause and Weis 1991). The F_v/F_m in the LDLT treatment decreased most, indicating that the drop in F_v/F_m was mainly caused by low non-freezing temperatures (Öquist et al. 1987, Smillie et al. 1988, Krause 1994). These results agree with former studies conducted with Scots pine (Ottander et al. 1995) and Norway spruce (Lundmark et al. 1998) needles. In this respect, a significant increase in the relative proportion of lipids as well as in the degree of desaturation of the fatty acids of the lipids has been observed in the thylakoid membranes of conifer chloroplasts (Öquist 1982, Senser and Beck 1982). Such changes indicate an increase in membrane fluidity and, as a consequence, also in membrane stability, in particular at low temperatures. Taking into account the quality of light is critical when comparing results from different studies. Such studies have indicated that, in addition to low temperature, short-day photoperiods also had a strong influence on the photochemical efficiency of needles (Vogg et al. 1998a,b).

In Study IV there was no correlation between the F_v/F_m of the non-frost-exposed needles and their FH, suggesting that the F_v/F_m of the non-frost-exposed needles was not a good indicator of their FH. Further work is needed to determine the interactions between temperature and photoperiod both under natural field conditions and in artificial controlled conditions.

5.5 Dry matter content and frost hardiness

The DM content of stems and needles started to increase before the onset of frost hardening (II, III and IV). When the DM content increased to a certain level, the FH increased quickly, depending on the organ. These observations agree closely with previous findings (Junttila and Kaurin 1990, Toivonen et al. 1991, Sutinen 1992, Repo et al. 1997, Ögren 1999a,b). The reduction in the water content is probably one of the first reactions at the moment of growth cessation and it is a reaction to physiological and structural changes in cells preceding cold acclimation (Repo et al. 2000a). Tissue dehydration and decreased water content can be ascribed to an increased DM content resulting from a thickening of the cell wall, lignification of xylem and accumulation of cryoprotectants in cells (Olien and Smith 1981, Sennerby-Forsse and von Fircks 1987, Sutinen 1992, Repo et al. 1997). The extent

of this reduction varies from species to species and from tissue to tissue (Kincaid and Lyons 1981, Smit-Spinks et al. 1984, Toivonen et al. 1991, Repo et al. 1997).

In Study II, the predictive power of the DM content of stems was variable and an increase in DM at low values had no effect on the FH. In Study III, there were no significant differences in the DM content of needles from different origins, whereas in Study IV the DM content of SDHT treatment in needles was lower than that in LDLT and SDLT treatments, which was the reverse of the development of FH in the needles. These results indicate that the DM content does not directly relate to the FH (Repo et al. 2000a).

5.6 Sugar concentrations and frost hardiness

In all of the three treatments in Study IV, the sugar concentration was highest in the needles, while it was intermediate and lowest in the stems and roots, respectively. These results agreed with the values obtained for FH in the same organs. In a study produced by Schaberg et al. (2000) of red spruce (*Picea rubens* Sarg.) seedlings, the needles generally contained the highest concentration of carbohydrates in comparison with the stems and roots, and they also exhibited the greatest seasonal change in carbohydrate concentration. In Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco.) in particular, the rapid rise in needle sugar concentration during cold acclimation coincided with a rapid increase in needle FH (Tinus et al. 2000). During cold acclimation, reductions in the strength of distal carbohydrate sinks limit the phloem transport away from the foliage and result in a build-up of foliar carbon reserves. Cold-induced reductions in the phloem transport itself may also contribute to the accumulation of foliar sugars (Wardlaw and Bagnall 1981, Grusak and Minchin 1989, Schaberg et al. 2000).

On the other hand, although many studies have documented an accumulation of sugars in woody plants in the autumn, this process has rarely been linked quantitatively with frost hardening (Ögren 1999a,b). The maximum concentrations of carbohydrates and maximum FH have not been observed simultaneously (Palonen 1999). The frost hardening is associated with changes in the accumulation of sugars. However, the mechanism underlying the development of frost hardening still remains to be elucidated (Greer et al. 2000).

5.7 Modelling of cold acclimation

The FH of roots, stems and needles in Scots pine seedlings reached a stationary level, or an asymptotical stationary level, when the environmental factors remained constant (IV). This supports the hypothesis that a discrete stationary level of FH prevails for those environmental factors. Similarly, in a number of other species, including *Pseudotsuga menziesii* (Timmis and Worrall 1975), *Picea abies* (Aronsson 1975, Christersson 1978), *Cornus stolonifera* (Harrison et al. 1978) and *Solanum acaule* and *S. commersonii* (Chen and Li 1976), FH has also been shown to increase at constant temperatures within a certain phase. When the photoperiod was held constant, it was apparent that seedlings developed a discrete level of FH that was stable over a considerable length of time (Greer et al. 1989). In the present study, the increase in the sugar concentration in the stems, needles and roots reached a stationary level during the treatments. For the stems and needles the moment at which the sugar concentration reached a stationary level was earlier than in the case of the FH, while for the roots it seems to occur simultaneously with frost hardening. These results show that under particular environmental conditions the sugar concentration may reach a certain level which may bring about efficient hardening. Accordingly, it may be speculated that the stationary level or the asymptotically approaching stationary level of frost hardiness is possibly connected with the sugar concentration.

The additive effect of photoperiod and temperature on FH was scarcely supported by all of the organs (IV). A consequence of such a hypothesis would be that the degree of hardiness is highest in the case of the SDLT treatment, where the low temperature and short photoperiod were assumed to cause their additive effects independently. In the stationary stage (steady state) or the asymptotical stationary state none of the organs followed that principle.

The results of Study IV suggest that, instead of using the principle of additivity, a more accurate estimation of the effect of temperature and photoperiod on FH could be obtained if new coefficients were introduced which would take into account the proportional effects of each environmental factor separately (Eq. [1], cf. Leinonen 1996).

[1] $$\hat{R}(t) = \hat{R}_{\min} + C_R \cdot (\alpha \cdot \Delta \hat{R}_T + \beta \cdot \Delta \hat{R}_P)$$

where $\hat{R}(t)$ is a stationary level of FH. $\hat{R}_{\min}$ is the minimum level of FH with no hardening induced by environmental factors, $\Delta\hat{R}_T$ is the increase in FH induced by temperature, and $\Delta\hat{R}_P$ is the increase in FH induced by the photoperiod. C_R is hardening competence (Fig. 1). The hardening competence is low (C_R=0) in the active growth phase and it increases to 1 towards the end of lignification phase (Leinonen 1996). α and β are coefficients specific to organs and they range between 0 and 1. For example, when the FH of an organ is driven solely by temperature, the coefficients would be α=1 and β=0, as the case of roots in Study IV, and when driven by photoperiod the coefficients would be α=0 and β=1.

During the photoperiod and temperature treatments in Study IV, the frost hardening of the roots was mainly driven by low temperature. At the start of the treatments the FH of the stems was mainly driven by low temperatures, while that of the needles was predominantly affected by a short photoperiod. These observations are in accordance with previous results, i.e. the hardening of the needles was induced by short days, but the stems and woody roots responded to low temperature (Burr et al. 1989, Aho 1994, Ryyppö et al. 1998). The results of many previous studies show that root cold acclimation and growth cessation are induced by low temperatures, e.g., *Picea glauca*, *Picea mariana*, *Picea sitchensis*, *Pinus sylvestris*, *Pseudotsuga menziesii*, *Larix kaempferi*, *Malus*, *Juniperus chinensis* "Hetzi", *Rhododendron* species, and *Alnus* (Pellett and White 1969, Wildung et al. 1973, Alexander and Havis 1980, Tremblay and Lalonde 1987, Bigras et al. 1989, Bigras and D'Aoust 1990, 1992, 1993, Mckay 1994). However, many other research studies have also suggested that both low temperature and short photoperiod affect the FH of roots. For example, Johnson and Havis (1977) have demonstrated the influences of both photoperiod and temperature on the hardening of secondary mature roots of *Picea glauca* (Moench) Voss. Colombo et al. (1995) have indicated that black spruce (*Picea mariana* (Mill.) B.S.P.) seedling roots increase their FH in a short photoperiod without cold temperature exposure. The results suggest that the model Eq. [1], which included temperature and photoperiod as input variables with the coefficients α and β, was more accurate than the models that use either temperature as the only input variable or temperature and photoperiod as input variables without α and β to predict the development of FH in different environmental conditions.

To obtain a better insight into the interaction between temperature and photoperiod, it will be necessary to submit plants to a greater range of photoperiod and temperature (Tremblay and Lalonde 1987) during various different phases of tree development. The relative contribution of photoperiod-induced and temperature-induced hardening in natural environments needs to be more clearly defined (Greer 1983). To develop the model further, experiments are also needed to estimate the model parameters for different pine origins and to examine the application potential of the model for other tree species.

6 CONCLUSIONS

The growth cessation and frost hardening of stems and needles of origins from different latitudes are related at the population level, i.e. early growth cessation predicts the early initiation of frost hardening. The coefficient of determination is higher for shoots than needles. There are differences in the timing of growth cessation between the northern and southern origins, and between the northern and intermediate origins. No constant temperature sums are found for the growth cessation of origins. Needles develop greater frost hardiness than buds, stems and roots.

Impedance spectroscopic analysis is a useful method for studying cold acclimation. The method is fast, and with proper planning over 300 samples can be measured in a single day, which is an advantage over most other methods. The relaxation time τ_1 of the non-frost-exposed stems of Scots pine shows a high correlation with their FH. The parameter τ_m and r_i in the non-frost-exposed needles will be useful for understanding the changes in the cell membranes, and also for understanding FH in the pre-hardening and hardening phases.

The main factor affecting the drop in the maximum photochemical efficiency of the photosystem II (F_v/F_m) of the non-frost-exposed Scots pine needles during hardening is a low non-freezing temperature. The F_v/F_m of the non-frost-exposed needles and the DM content of different organs are not considered to be reliable methods for determining FH.

The sugar concentration in stems and roots of LDLT and SDLT treatments increased during the hardening, whereas in the case of the SDHT treatment it was constant, suggesting the finding that the frost hardiness of these two organs was affected mainly by

temperature. The sugar concentration in the different organs followed the sequence needles > stems > roots, which also matched the levels of frost hardiness.

The effects of both photoperiod and temperature are important for the development of the FH of Scots pine, and including these factors in the mathematical model increases its reality. The additive models need revision since the hardening responses displayed by the factors are dependent on organ, and there is an interaction in the hardening responses to temperature and photoperiod.

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ORIGINAL ARTICLE

Tapani Repo · Gang Zhang · Aija Ryyppö
Risto Rikala · Martti Vuorinen

The relation between growth cessation and frost hardening in Scots pines of different origins

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Abstract The cessation of shoot elongation, diameter growth and needle elongation were compared with the initiation of frost hardening of the stems and needles in an 8-year-old provenance trial of Scots pine (*Pinus sylvestris* L.) established in central Finland. The saplings were of six different origins ranging from Estonia to northern Finland, forming a latitudinal gradient of ca. 10°N. The frost hardiness of the stems of current-year shoots was assessed by electrical impedance analysis and that of current-year needles by electrolyte leakage and visual scoring of damage. Artificial freezing tests were used in the assessments. The pattern of growth cessation (shoot and needle elongation, diameter growth) tended to follow the latitude of origin, i.e. growth ceased in the northernmost provenance first and in the southernmost one last. Both stems and needles of the northern provenances hardened earlier than the southern ones, but the differences in hardiness disappeared as hardening progressed. Growth cessation and initial hardening to –15°C were clearly correlated at the provenance level, indicating that growth must cease prior to hardening, and that earlier cessation of growth predicts earlier frost hardening of stems and needles. No differences in frost hardiness of stems were found at the provenance level at the end of the growing period in August. At that time, the frost hardiness of needles of the northernmost provenance was higher than that of other origins. Within the provenance, the stems were less hardy than the needles.

Key words Cold acclimation · Frost hardiness · Growth cessation · *Pinus sylvestris* · Provenance

T. Repo (✉) · G. Zhang · A. Ryyppö
Faculty of Forestry, University of Joensuu, P.O. Box 111, 80101 Joensuu, Finland
e-mail: tapani.repo@joensuu.fi

R. Rikala · M. Vuorinen
Finnish Forest Research Institute, Suonenjoki Research Station, Juntintie 40, 77600 Suonenjoki, Finland

Introduction

Among the pine species, Scots pine (*Pinus sylvestris* L.) has the widest natural range of distribution. Accordingly, variation in climatic conditions over the distribution range of Scots pine is great (Mirov 1967). Owing to the long adaptation time, the phenology and the frost hardiness of native origins is generally well synchronized with the weather conditions of the local growing site. In that synchronization, two adaptive traits, the timing of growth cessation and initiation of frost hardening have key roles.

Forest populations of Scots pine differ in the timing of hardening: northern origins initiate frost hardening earlier than southern ones (Dormling et al. 1977; Nilsson and Eriksson 1986; Aho and Pulkkinen 1991; Toivonen et al. 1991; Aho 1994; Sundblad and Andersson 1995; Hurme et al. 1997). Independent of the origin, cessation of growth and induction of the dormant phase are required for the development of the maximum level of hardiness (Dormling 1993). However, the quantitative relationship between growth cessation and frost hardiness in different origins of Scots pine is not well known. Previously, a close relationship was found between bud set and frost hardening in first-year seedlings of Scots pine (Hurme et al. 1997); however, little is known about the relation in older seedlings and saplings (Repo et al. 2000). In order to adapt silviculture to the present and predicted climate, it is essential, for example, for provenance transfers to know how growth cessation is related to frost hardening in different tree species and origins and how they are related to seasonal changes in temperature and photoperiod.

The aim of this experiment was to study the cessation of stem and needle elongation and diameter growth of the stem in relation to frost hardening of these organs of Scots pine saplings in a provenance field trial established in central Finland. Furthermore, the methods for assessing frost hardiness were compared.

Table 1 Latitude, longitude and mean effective temperature sum ($\Sigma T_{+5°C}$) (in degree days) (threshold of daily mean temperature +5°C) of the seed origins (mean of 30 years) (Nerg et al. 1994)

Origin		Latitude (N)	Longitude (E)	$\Sigma T_{+5°C}$, d.d.
1	Saarenmaa, Estonia	58°22′	22°51′	1450
2	Tenhola, Finland	60°03′	23°04′	1300
3	Korpilahti, Finland	62°00′	25°28′	1100
4	Kinnula, Finland	63°32′	25°03′	1000
5	Suomussalmi, Finland	65°10′	29°05′	800
6	Muonio, Finland	67°56′	23°34′	700

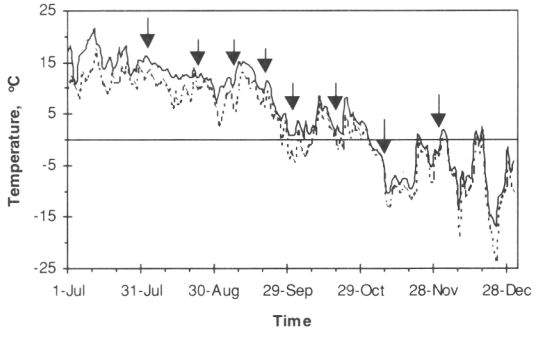

Fig. 1 Daily mean (*continuous line*) and minimum (*dotted line*) air temperature at the provenance trial (Suonenjoki, Finland) during the study period. The *arrows* indicate the time of frost hardiness assessment

Materials and methods

Plant material

This study was based on an 8-year-old field provenance trial of Scots pine (*Pinus sylvestris* L.) established in the Research Nursery at Suonenjoki Research Station of the Finnish Forest Research Institute (FFRI) (62°39′N, 27°03′E, 130 m above sea level (a.s.l.)). The seeds for the trial were collected from six open-pollinated natural stands (five in Finland and one in Estonia) representing the north-to-south climatic gradient (Table 1). The bareroot 2+0 seedlings grown in Suonenjoki nursery (for details see Nerg et al. 1994) were planted on an old nursery field in spring 1993. The soil in the nursery field was a mixture of fine sand and peat (<3% w/w). Two hundred seedlings from each origin were planted in one block with a spacing of 1.0×0.5 m between seedlings. The sample trees selected for the study corresponded to the mean height of the respective provenance.

The air temperature was recorded close to the provenance trial, 2 m above ground level at Suonenjoki Research Station (Fig. 1). The daily mean temperature sums of the seed origins were calculated according to the temperature records at the nearest meteorological stations of the origins, which were averages of 30 years.

Measurement of shoot growth

Growth of the saplings was monitored from 11 May to the end of August 1998. Elongation of the current-year shoots (for each origin n=10), diameter growth of the current-year shoots (n=10 per origin) and elongation of the current-year needles (n=100 per origin) were measured weekly. Growth cessation was determined as the date when the mean shoot and needle elongation and the mean diameter growth reached 90% of their final levels.

Freezing tests

Frost hardiness was studied from 3 August to 5 December 1998. Sixteen sampling trees from each of the six provenances were selected. Current-year lateral shoots were sampled at 2- to 3-week intervals. The freezing tests were carried out at Suonenjoki Research Station and the Faculty of Forestry, University of Joensuu.

Immediately after harvesting, eight stems (16 in the last two tests) and 52 needles from each origin were prepared for each exposure temperature (6–7 freezing temperatures and control +3°C). The samples were placed into plastic bags and a little distilled water was sprayed into the bags to prevent excessive supercooling before initiation of the exposure in air-cooled chambers. To cover the assumed critical temperature range, the test temperatures were chosen on the basis of a previous freezing test. The temperature range used in the freezing tests was from 0 to –130°C. The programmed initial and end temperature of the exposure was 3°C; the samples were kept at the target temperature for approximately 4 h. The rate of cooling and warming was 5°C h^{-1}. After the freezing test, samples were used immediately for measurements of freezing tolerance by the methods of electrical impedance and electrolyte leakage. Visual damage on needles was scored 1 week after the exposure to frost.

Assessment of frost hardiness

Electrical impedance analysis of stems

Assessment of frost hardiness by the electrical impedance analysis is based on the decrease of the extracellular resistance due to damage in plasma membrane and consequent leaking of the intracellular ions into the extracellular space. Even though the method is destructive, it is an in situ measurement and it gives a primary measure of frost damage in cells. In this method, alternating current (AC) is applied to a piece of plant tissue. The proportion of current going through the extracellular and intracellular space of a tissue is dependent on the AC frequency and the tissue properties. The tissue features (e.g. extracellular resistance) can be quantified by the equivalent circuit analysis (Repo et al. 1994; Ryyppö et al. 1998).

The impedance spectra of stems (2–4 mm in diameter) were measured with an Ag/AgCl cell (RC1, WPI Ltd., Sarasota, USA) connected to a HP 4284A LCR meter (Hewlett-Packard, Santa Clara, USA) (Repo 1994; Repo et al. 1994). For impedance analysis, a 15-mm section was cut from the middle portion of the stem soon after the freezing test. The section was placed between the electrode pastes of the Ag/AgCl cell to measure an impedance spectrum at 42 frequencies between 80 Hz and 1 MHz (Repo 1994). The input voltage level of the sine signal was 100 mV (root mean square).

The impedance spectra were modelled using the double-distributed circuit element (DCE) model for stems (Repo et al. 1994) from the beginning of the experiment until 7 September. Thereafter, owing to the disappearance of the low frequency arc at most test temperatures, a single-DCE model was used instead of the double-DCE. The parameters of the equivalent circuits were estimated with an automated complex non-linear least squares (CNLS) fitting program LEVM v.6.0 (J.R. Macdonald, Department of Physics and Astronomy, University of North Carolina, Chapel Hill, NC, USA), which has been further developed and automated by Dr. Repo and

coworkers. Extracellular resistance (R_e) was obtained by the double-DCE model as $R_e=R+R_1+R_2$ and by the single-DCE model as $R_e=R+R_1$, where R, R_1 and R_2 are estimated resistances. For the determination of frost hardiness, the specific extracellular resistance (r_e) was calculated as (Repo et al. 1994)

$$r_e=A_c/L\times R_e \quad (1)$$

where r_e is the specific extracellular resistance, A_c is the cross-sectional area of the stem, which is considered to be a circle ($A_c=\pi d^2/4$, d is the diameter of the stem), and L is the length of the sample (L=15 mm).

To obtain the frost hardiness, the specific extracellular resistance (units Ω m) was modelled by a logistic sigmoid function (in Eq. 2) with respect to the treatment temperature

$$y(x)=A/(1+e^{B(C-x)})+D \quad (2)$$

where y is parameter r_e, x is treatment temperature, B is the slope at inflection point C, C is frost hardiness, and A and D determine the asymptotes of the function (Repo et al. 1994,1997).

Electrolyte leakage test of needles

As a consequence of frost damage to the plasma membrane, electrolytes leak from the symplast to the apoplast as the primary symptom of cellular damage. This leakage can be detected after 'washing' the electrolytes from the apoplast into distilled water and by measuring the electrical conductivity of the water solution. The conductivity of the heat-killed samples gives a measure of the total amount of tissue electrolytes. Frost injuries in cells may be determined by comparing the relative conductivities between the non-frozen and frozen samples (Flint et al. 1967; Burr et al. 1990; Sutinen et al. 1992).

After freezing treatment, 32 needles of the shoots from each freezing temperature for each provenance were selected for the electrolyte leakage test. The samples, 10 mm in length, were cut from the middle of the needles. The samples were rinsed with distilled water and put into test tubes (eight samples per tube) as four replicates. Distilled water (6 ml) was added to each test tube, which was then shaken at room temperature for 24 h before the first conductivity measurement (L_1). The samples were then heat killed at 92°C for 20 min and shaken another 24 h before the second conductivity measurement (L_2). The relative electrolyte leakage (REL) was defined as

$$REL=(L_1/L_2)\times 100 \quad (3)$$

Frost hardiness was estimated as the inflection point (parameter C) of Eq. 2, with y being the REL.

Visual scoring of damage of needles

Frost hardiness assessment of needles by visual scoring of damage is based on the estimation of chlorophyll breakdown as the browned needle area. Since the scoring takes place after 1 week of the freezing test, it indicates the primary frost damage and consequent secondary damage on plant cells.

After freezing treatment, 20 needles per treatment temperature from each provenance were used for visual scoring of damage. The needles were put on moistened paper in petri dishes and transferred to a growth chamber [23°C; photon flux density (PFD): 100 μmol m^{-2} s^{-1}]. After 1 week, damage was scored. The scores were converted to binary numbers, i.e. if the damage was <20%, the score became 1, otherwise it became 0. The frost hardiness was obtained by interpolation and defined as the effective temperature where 50% of the needles were affected (LT_{50}).

Statistical analysis

One-way analysis of variance (ANOVA) was used to calculate the data on cessation of growth and frost hardiness at different times

Table 2 Frost hardiness (LT_{50}) of current-year stems (A) and needles (B, C) of 8-year-old Scots pine saplings of six provenances (see Table 1). The differences between provenances in the LT_{50} estimates on each time instant are taken as significant and marked with *different letters* (in rows) when the values of the Wald 95% confidence intervals do not overlap. *EI* refers to electrical impedance method, *EL* to electrolyte leakage method and *VD* to visual scoring of damage

Date	Provenance (southern→northern)					
	1	2	3	4	5	6
A. Stem (EI)						
3 Aug	–5.1*	–5.8*	–5.8*	–6.5a	–4.9a	–5.8*
24 Aug	–5.0a	–5.3a	–5.5a	–5.3a	–5.9*	–5.9*
7 Sep	–7.4ab	–6.0a	–9.7bc	–8.9bc	–11.0c	–10.6c
21 Sep	–5.9a	–9.3ab	–13.4bc	–9.5abc	–13.1bc	–15.0c
5 Oct	–19.3a	–25.3ab	–24.6ab	–28.2ab	–35.0bc	–46.1c
19 Oct	–18.4a	–28.7a	–26.3a	–27.1a	–29.0a	–28.6a
9 Nov	–41.4ab	–38.5ab	–31.6*	–33.0a	–45.7b	–48.6b
30 Nov	–38.1a	–40.3a	–45.7a	–56.6a	–42.5a	–54.8a
B. Needle (EL)						
3 Aug	–5.9*	–5.8*	–5.2a	–5.6a	–5.6a	–6.6b
24 Aug	–5.4a	–4.5a	–5.1a	–5.2a	–5.5a	–6.9b
7 Sep	–8.0a	–7.7a	–9.1*	–10.4b	–12.0*	–13.8c
21 Sep	–12.8a	–12.1a	–15.3b	–16.5c	–21.7d	–25.6e
5 Oct	–28.3a	–28.7a	–35.7b	–34.8b	–46.3c	–55.2c
19 Oct	–37.3a	–36.5a	–58.5c	–51.8*	–59.5bc	–48.6b
9 Nov	–90.8a	–86.5a	–91.5a	–81.3a	–97.4a	–89.7a
C. Needle (VD)						
3 Aug	–5.0	–5.0	–5.0	–6.9	–5.4	–7.2
24 Aug	–3.1	–5.0	–5.5	–5.5	–6.7	–7.8
7 Sep	–7.3	–6.7	–7.9	–9.8	–10.8	–12.8
21 Sep	–11.3	–10.5	–12.6	–12.6	–15.0	–13.7
5 Oct	–21.6	–23.1	–28.8	–30.0	–31.3	–37.5
19 Oct	–19.0	–25.0	–32.0	–30.0	–53.0	–45.0
9 Nov	–52.8	–50.9	–56.8	–75.6	–62.4	–68.6
30 Nov	<–100	<–100	<–100	<–100	<–100	<–100

*The Wald 95% confidence intervals were infinite

of measurement. Regression analysis was applied for analysing the correlation between frost hardiness of needles by electrolyte leakage and visual damage scoring, and for analysing the relationship between frost hardiness of stems and needles. Paired samples *t*-test was used for multiple comparison of the frost hardiness at different times of measurement. Wald's χ^2-test was used for comparison of provenances at different times.

The relationship between cessation of growth and initiation of frost hardening at the provenance level was determined by comparing dates of growth cessation of stems (elongation and diameter growth) and needles with dates when a frost hardiness level of –10°C and –15°C was reached in the respective organs. The relationship between the traits was tested with linear regression analysis.

Results

Frost hardiness of stems

In August, the frost hardiness of stems (between –4.9°C and –6.5°C) did not differ significantly between provenances; however, from the beginning of September until early October the relationship was obvious (Table 2). Frost hardening was initiated in September, first in the northern provenances, then in the intermediate provenances and finally in the southern provenances. From the beginning of September to early October in the two northernmost provenances frost hardiness increased sharply: from –11.0°C to –35.0°C in provenance 5 and from –10.6°C to –46.1°C in provenance 6 (Table 2). During that period, the rate of hardening was 4.0°C week^{-1} and 8.5°C week^{-1} for these two northern provenances, respectively, whereas the rate of hardening for the southernmost provenance (provenance 1) was 2.8°C week^{-1}. The greatest difference in frost hardiness (27°C) between the northernmost and the southernmost provenances was found in early October. Hardening stopped for a while in October, and in four provenances it even reversed. The maximum frost hardiness ranged from ca. –38°C (southern origin) to –55°C (northern origin) without significant differences between origins at the end of November.

Frost hardiness of needles

The frost hardiness of needles assessed by the electrolyte leakage method showed no differences between provenances 1–5 in August, but needles from the northernmost provenance (6) were more hardy than those from the other provenances (Table 2). Frost hardening was initiated in the beginning of September. The needles of northern provenances hardened faster than those of the southern provenances. The most significant differences between provenances were found from late September to the beginning of October, and during that time the largest difference between origins was 27°C. From mid-October until the end of the experiment, the frost hardiness of all provenances, especially the southern ones, increased significantly, indicating that the southern provenances achieved their fastest rate of hardening later than the northern ones did. At the end of November, all the provenances gained about the same level of hardiness, ranging from –80°C to –90°C. In the assessment on 30 November, the electrolyte leakage method failed to give reliable estimates of frost hardiness.

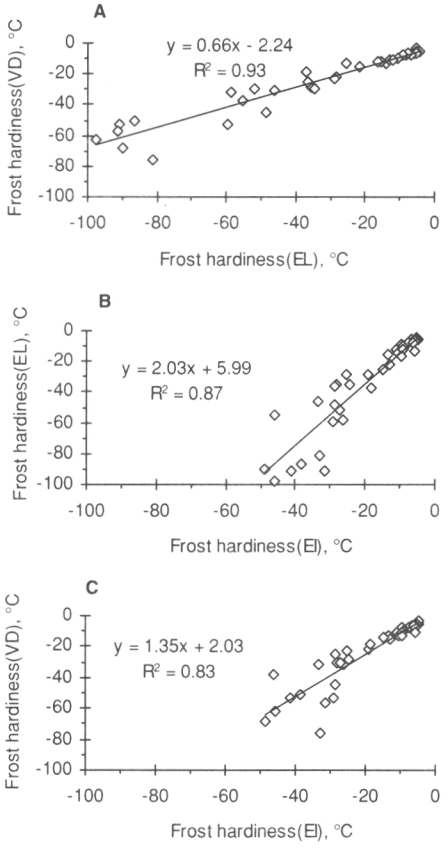

Fig. 2A–C Relationship between frost hardiness of stems and needles: comparison of organs and methods. **A** Visual damage scoring of needles (VD) versus electrolyte leakage of needles (EL); **B** EL versus electrical impedance analysis of stems (EI); **C** VD versus EI. Pooled data of six provenances. The best fit regression line and equation with the coefficient of determination is indicated

According to visual scoring of damage, in August the needles in the northernmost provenance (6) were hardier than those in the southernmost provenances (Table 2). From late September until early October the frost hardiness of all provenances increased with high rates. The northern provenances hardened faster than the southern ones did, e.g. 12°C week^{-1} and 5°C week^{-1} for the northernmost and the southernmost provenance, respectively. The largest difference in frost hardiness was 34°C, which occurred between provenances 5 and 1 on 19 October. Temporarily there was a slowing of hardening, even dehardening, on 19 October, but the phenomenon was not as clear as in stems. At the end of November, the frost hardiness of needles of all provenances was higher than

Table 3 Comparison of frost hardiness of different organs and methods at different times at the provenance level (*t*-test). *EL* refers to electrolyte leakage method, *VD* to visual scoring of damage and *EI* to electrical impedance analysis

Date	Paired difference of means	*t*	df	Sig. (2-tailed)
A. Needles (EL) vs needles (VD)				
3 Aug	–0.01	–0.03	5	0.978
24 Aug	0.17	0.34	5	0.750
7 Sep	–0.96	–8.32	5	0.000***
21 Sep	–4.70	–2.87	5	0.035*
5 Oct	–9.46	–4.23	5	0.008**
19 Oct	–14.71	–4.02	5	0.010**
9 Nov	–28.33	–5.50	5	0.003**
B. Stems (EI) vs needles (EL)				
3 Aug	0.11	0.36	5	0.732
24 Aug	0.04	–0.15	5	0.885
7 Sep	1.22	2.39	5	0.063
21 Sep	6.34	4.63	5	0.006**
5 Oct	8.67	6.35	5	0.001***
19 Oct	22.36	6.13	5	0.002**
9 Nov	49.73	19.88	5	0.000***
C. Stems (EI) vs. needles (VD)				
3 Aug	0.10	0.30	5	0.775
24 Aug	0.13	0.26	5	0.808
7 Sep	0.26	0.48	5	0.649
21 Sep	1.64	1.60	5	0.171
5 Oct	–0.79	–0.42	5	0.692
19 Oct	7.66	1.79	5	0.134
9 Nov	21.40	4.53	5	0.006**

Two-tailed significance: *$P<0.05$, **$P<0.01$, ***$P<0.001$

–100°C; most of the needles treated at a temperature of –130°C survived.

Although the electrolyte leakage and visual damage scoring of needles gave different estimates for frost hardiness, these estimates were linearly correlated (Fig. 2A). When the data of all provenances were pooled, in August there were no differences between the two methods. On the other hand, there were significant differences in estimated frost hardiness later, especially on 7 September (Table 3). Typically, the frost hardiness of needles assessed by the electrolyte leakage method was higher than that obtained by scoring visual damage.

Comparison of frost hardiness between stems and needles

Frost hardiness of stems and needles was about the same from early August to the beginning of September. Afterwards, the frost hardiness of the needles increased faster than that of the stems (Table 3). At the end of the experiment, needles were much hardier than stems: in November the difference was 50°C by electrolyte leakage and 21°C by visual damage scoring of needles, which both were statistically significant (Table 3). There was a clear linear relationship between the frost hardiness of stems and needles (Fig. 2B,C; Table 3).

Relationship between growth cessation and frost hardening

Growth ceased first in the northern provenances and last in the southern ones. Shoot elongation of the northern-

Table 4 Dates of cessation of shoot elongation (A), stem diameter growth (B) and needle elongation (C) of Scots pine of different provenances (see Table 1) (90% from the final length reached) with respective temperature sums ($\Sigma T_{+5°C}$), and the dates for reaching frost hardiness (*FH*) of –10°C and –15°C in stems and needles. "Duration" refers to time from cessation of growth to hardening to –10°C and –15°C

Provenance	Date of cessation of growth	$\Sigma T_{+5°C}$ (d.d.)	Date of FH to		Duration, days	
			–10°C	–15°C	–10°C	–15°C
A. Shoot elongation and hardening						
1	4 Jul	444	26 Sep	1 Oct	85	90
2	3 Jul	439	22 Sep	27 Sep	82	87
3	2 Jul	426	8 Sep	26 Sep	69	87
4	1 Jul	413	22 Sep	24 Sep	84	86
5	29 Jun	389	4 Sep	23 Sep	68	87
6	24 Jun	334	5 Sep	21 Sep	74	90
B. Stem diameter growth and hardening						
1	13 Aug	869	26 Sep	1 Oct	45	50
2	14 Aug	876	22 Sep	27 Sep	40	45
3	14 Aug	876	8 Sep	26 Sep	26	44
4	7 Aug	819	22 Sep	24 Sep	47	49
5	2 Aug	769	4 Sep	23 Sep	34	53
6	28 Jul	718	5 Sep	21 Sep	40	56
C. Needle elongation and hardening						
1	3 Aug	780	13 Sep	24 Sep	42	53
2	31 Jul	748	13 Sep	24 Sep	45	56
3	1 Aug	758	8 Sep	21 Sep	39	52
4	3 Aug	780	5 Sep	18 Sep	34	47
5	29 Jul	727	2 Sep	12 Sep	36	46
6	18 Jul	614	30 Aug	9 Sep	44	54

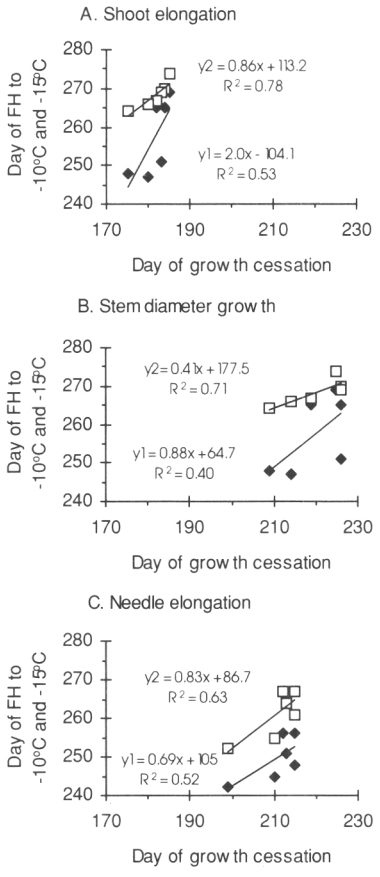

Fig. 3A–C Relationship between day of year of frost hardening (FH) to –10°C (◆) and –15°C (❑) and day of year of growth cessation (indicated by y1 and y2 in the regression lines, respectively) for different provenances. **A** Frost hardening of stems and cessation of shoot elongation; **B** frost hardening of stems and cessation of diameter growth; **C** frost hardening of needles and cessation of needle elongation. The best fit regression lines and equations with the coefficient of determination are indicated

most provenance (6) ceased on 24 June 1998, i.e. 9–10 days earlier than that of the more southern provenances (Table 4). Diameter growth of the stem and needle elongation of the northernmost provenance (6) stopped on 28 July and 18 July, respectively, i.e. 16 days earlier than those of the southernmost provenance. The difference in temperature sum at the time of cessation of shoot elongation was 110 degree days (d.d.) between the northern and southern origins (Table 4). These phenomena were in accordance with the timing of initial hardening to –10°C and –15°C: the northernmost provenance (6) reached a frost hardiness of –10°C and –15°C 21 and 10 days earlier than the southernmost provenance (1), respectively (Table 4). A similar situation was also found for needles, i.e. cessation of needle elongation and initial hardening both occurred earlier in the northernmost provenance (6) than in the southern provenances (Table 4).

When comparing growth cessation and initiation of frost hardening of Scots pine origins, a linear correlation was obtained at the provenance level (Fig. 3). The correlation between growth cessation (shoot elongation, stem diameter growth and needle elongation) and hardening to –15°C was higher than between growth cessation and hardening to –10°C (Fig. 3).

Discussion

Growth cessation of different origins

Shoot elongation ceased at the end of June and at the beginning of July long before cessation of needle elongation and diameter growth in accordance with previous studies (see Huikari and Paarlahti 1967; Raulo and Leikola 1974; Parviainen 1975; Koski and Sievänen 1985). There were great differences in the timing of growth cessation between the northern and southern origins, and between the northern and central origins. These results agree with those obtained by Oleksyn et al. (1992) in their study of growth in different European Scots pine populations under different photoperiods, where a strong correlation existed between timing of growth cessation and latitude of the origin. A north-to-south latitudinal trend has also been found in the timing of height growth period in other tree species, e.g. in *Picea abies* (Heide 1974; Junttila and Skaret 1990), *P. glauca* (Junttila and Skaret 1990), *Acer saccharum* (Kriebel 1957) and *Salix pentandra* (Junttila and Kaurin 1985). In the present study, the difference in the date of growth cessation and temperature sum between central and southern origins (from 58°N to 63°N) was small, however.

Growth cessation of Scots pine saplings was related to the temperature sum (in d.d.) in accordance with previous studies (Wright 1976; Koski and Sievänen 1985). However, the southern provenances required some higher temperature sums for growth cessation than the northern ones, in contrast to the results of Koski and Sievänen (1985). Accordingly, it might be possible that the photoperiod or the joint effect of the photoperiod and temperature have some impact on growth cessation in 8-year-old saplings, or provenances have different thresholds of temperature sum for growth cessation. The first-year seedlings of Scots pine display a joint effect of temperature sum and photoperiod for growth cessation (Koski and Selkäinaho 1982; Koski and Sievänen 1985; Oleksyn et al 1992).

Frost hardiness between origins

In both stems and needles, the frost hardiness of different provenances in August was between –5°C and –8°C. At the end of the growing season, the frost hardiness of

stems and needles varied between –5°C and –8.5°C for first-year seedlings (Hurme et al. 1997) and from –4°C to –5°C for second-year seedlings (Rikala and Repo 1987; Repo et al. 1994; Ryyppö et al. 1997). Usually the origin has not been found to affect frost hardiness during the growing season (Aho and Pulkkinen 1991; Toivonen et al. 1991), as was also shown in the present study for the stems but not in needles (3 and 24 August, Table 1).

Hardening was initiated earlier in the northern than in the southern origins, and a negative relationship between the frost hardiness and the latitude of origin was found during the hardening. This is in accordance with previous studies on first-year (Dormling et al. 1977; Nilsson and Eriksson 1986; Aho and Pulkkinen 1991; Toivonen et al. 1991; Aho 1994; Sundblad and Andersson 1995; Hurme et al. 1997) and older seedlings and trees of Scots pine (Lindgren and Nilsson 1992; Dormling 1993; Andersson 1994; Nilsson and Walfridsson 1995; Beuker et al. 1998), and on other tree species, such as *Picea abies* (Ekberg et al. 1979), *Pinus contorta* (Hagner 1970; Jonsson et al. 1981) and *Picea glauca* (Simpson 1994). With fully hardened samples the effect of latitudinal origin became insignificant and all provenances of this study were hardy enough to tolerate the harsh winter conditions. This means that, in Scots pine, the maximum hardening potential would not be a limiting factor for south-to-north provenance transfers but rather the timing of frost hardening.

On the provenance level, the differences in the rate of hardening in early autumn were large. For both stems and needles, the maximum rate of hardening was higher in the northern than in the southern provenances. The time of maximum rate of hardening also occurred earlier in the northern than in the southern provenances. This may indicate that different provenances have their specific rates of hardening and maybe different stationary levels with respect to temperature and photoperiod (Leinonen et al. 1995; Leinonen 1996): when the photoperiod and the temperature reach a genotype-specific level, hardening is initiated according to the intrinsic attributes of genotypes. However, the hardening is not a one-directional process only, in that it may temporarily cease and even reverse, as found in this study.

Frost hardiness between stems and needles

The frost hardiness of stems and needles of the provenances first remained at a relatively constant level until late summer and then increased with an enhanced rate. The hardening temporarily ceased and even reversed on 19 October. Several studies have suggested that hardening occurs in stages (Weiser 1970; Kacperska 1985; Sakai and Larcher 1987); and in conifers particularly (Tumanov and Krasavtsev 1959; Glerum 1973,1985; Cannell and Sheppard 1982). The first stage is suggested to be driven by increasing night-length and the second stage by low temperatures. In our study, no first stage of hardening could be found but hardening proceeded quite fast when it commenced. The temporary interruption of the hardening in October falls in the temperature-driven phase, and it is probably due to a warm time lapse between 12 and 15 October when the daily mean temperature rose above 5°C.

Frost hardiness in trees works like an integrator; it does not respond immediately to short-term changes in the environment but according to a time constant (Repo and Pelkonen 1986). Thus, the occurrence of different phases of hardening may require that the rate of long-term change of driving factors takes place slow enough in regard to the rate of physiological and structural changes connected with hardening. Furthermore, hardening is non-linearly dependent on the driving factors (Leinonen 1996). If the driving factors stay approximately constant, for a while, or at a range not important for inducement of hardening, the frost hardiness may stabilize at a respective level too. Such occasions are seldom in the northern latitudes in autumn, however.

The stems of Scots pine were less hardy and hardened later than needles did (Tables 2 and 3). This is in accordance with results obtained with seedlings of Scots pine, where the stems hardened more slowly than needles in artificial conditions and frost hardening was deeper in the needles than in the stems (Hurme et al. 1997; Ryyppö et al. 1998). This means that the responses of stems and needlès to the changing environmental conditions differ during autumn. The delayed hardening of stems compared with needles might be due to the diameter growth in stems, which continued longer than the needle elongation.

Relationship between cessation of growth and frost hardiness

Cessation of both shoot and needle elongation and diameter growth took place well before the initiation of the steady hardening period. The correlation between the timing of growth cessation and hardening to –15°C was evident, more so for stems than for needles. This is in accordance with the results obtained between bud set and initiation of frost hardening of stems and needles of first-year Scots pine seedlings at the population level (Hurme et al. 1997), and for *Picea abies* (Johnson and Apeland 1988) and *Pseudotsuga menziesii* (Campbell and Sorensen 1973; Rehfeldt 1979; Loopstra and Adams 1989).

In conclusion, growth cessation and frost hardening of stems and needles of origins from different latitudes are related at the population level, i.e. the early growth cessation predicts the early initiation of frost hardening. The coefficient of determination was higher for shoots than needles. No constant number of temperature sums was found for growth cessation of origins. Needles became more frost hardy than stems.

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II

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The electrical impedance spectroscopy of Scots pine (*Pinus sylvestris* L.) shoots in relation to cold acclimation

T. Repo[1,4], G. Zhang[1,3], A. Ryyppö[1] and R. Rikala[2]

[1]*Faculty of Forestry, University of Joensuu, PO Box 111, FIN-80101 Joensuu, Finland*
[2]*Finnish Forest Research Institute, Suonenjoki Research Station, Juntintie 40, FIN-77600 Suonenjoki, Finland*

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Abstract

Electrical impedance spectroscopy (EIS) was applied to stems of Scots pine (*Pinus sylvestris* L.) in a provenance field trial during frost hardening to find an EIS parameter for assessing frost hardiness (FH) without a controlled freezing test. The FH of stems and needles assessed by controlled freezing tests was compared with the equivalent circuit EIS parameters of a distributed model of stems (not exposed to controlled freezing treatment) and with dry matter (DM) content of stems. Significant differences in the equivalent circuit parameters, FH and DM content were found between provenances. The relaxation time (τ_1), describing the peak of the high frequency arc of the impedance spectrum, and the intracellular resistance (r_i) of stems increased with increasing FH. According to the linear regression, the coefficient of determination (R^2) between the FH of stems and needles with τ_1 of the stem was 0.87 and 0.89, and with r_i of the stem 0.74 and 0.85, respectively. The relation between FH and τ_1 changed with the degree of hardiness. The highest coefficient of determination was 0.95 in September when the FH of needles, ranging from −10 °C to −25 °C, was predicted with an accuracy of ±2.0 °C. The resistance parameter r_2, describing the width of the low frequency arc of the impedance spectrum, decreased prior to and during the initial hardening: significant differences were found between provenances. This indicates that r_2 was not related to frost hardening *per se*. It is concluded that it is possible to distinguish the hardening patterns of different provenances by τ_1 in the rapid phase of hardening without controlled freezing tests.

Key words: Dry matter content, frost hardiness, impedance spectroscopy, relaxation time, Scots pine.

Introduction

An easy and fast method for the determination of frost hardiness (FH) in plants is still lacking. Several techniques are currently used that necessitate a controlled freezing test with several freezing temperatures. These techniques include the electrolyte leakage test, the visual scoring of damage, the measurement of chlorophyll fluorescence, differential thermal analysis, plasma membrane H^+-ATPase activity, and electrical impedance analysis (Timmis, 1976; Palta *et al.*, 1978; Glerum, 1985; Ryyppö *et al.*, 1998). Thus the FH assessment is time-consuming, requires expensive equipment, and also a considerable amount of material.

Electrical impedance spectroscopy is a method for studying the structure of organic and inorganic materials (Ackmann and Seitz, 1984; Foster and Schwan, 1989; Macdonald, 1992; Repo and Zhang, 1993; Repo *et al.*, 1997; Schwan, 1999). In this method, alternating current (AC) is applied to a piece of plant tissue. Alternating current causes polarization and relaxation in the sample leading to changes in amplitude and phase of the applied AC signal. According to those changes an impedance of the sample can be determined which is formed of a real and an imaginary part in a complex plane. When the real and imaginary part is measured at different frequencies an impedance spectrum is obtained.

The proportion of the current passing through the apoplastic and symplastic space of the tissue sample depends

[3]Current address: Department of Horticulture, Agricultural University of Hebei, Baoding, Hebei. 071001, P.R.China.
[4]To whom correspondence should be addressed. Fax: +358 13 2514444. E-mail: tapani.repo@forest.joensuu.fi

on the AC frequency and the tissue properties. Cell membranes have high impedance at low frequencies. Accordingly, the current flows in the apoplastic space which determines the total impedance. In the apoplastic space ions are the main current carriers. The impedance of the cell membranes decreases with increasing frequency. Accordingly the symplastic space become conductive and at high frequencies the symplastic and apoplastic resistance form a parallel circuitry.

Changes in cellular features appear in their EIS-characteristics. The tissue features can be quantified in EIS by the equivalent electrical circuit analysis (Zhang and Willison, 1992; Repo and Zhang, 1993; Repo *et al.*, 1994; Zhang *et al.*, 1995). With a proper equivalent electrical model it is possible to study the effects of different stress factors on the tissue properties according to the changes in the parameters of the model (Zhang and Willison, 1992; Zhang *et al.*, 1993; Repo *et al.*, 1994; Repo and Pulli, 1996; Ryyppö *et al.*, 1998).

It has been found that several equivalent circuit parameters of the distributed model for the stems of Scots pine undergo seasonal variation (Repo *et al.*, 1995, 1997). Some changes in the parameters coincided with FH, and the highest correlation was previously found between intracellular resistance and FH. It has been hypothesized that impedance analysis might be used for assessing FH without a controlled freezing test. In order to develop practical applications in nurseries and tree breeding, this hypothesis had to be tested in an experiment with genotypes, each with its own different hardening pattern. If there is a common relationship between FH measured by a conventional method and the selected parameter of a non-frost exposed sample, then the method could be used for FH assessment without using a controlled freezing test.

The aim of this study was to examine the relationship between the frost hardiness of stems and needles, assessed by controlled freezing tests, with equivalent circuit EIS parameters of stems not exposed to frost. The equivalent circuit EIS parameters and FH were compared with the dry matter content of the samples. The study took place by using a provenance field trial of Scots pine.

Materials and methods

Plant material

The study was based on a field provenance trial with six Scots pine (*Pinus sylvestris* L.) origins. The seeds for the trial were collected from six open-pollinated natural stands over an area ranging from Estonia to northern Finland. The bareroot 2+0 seedlings (see Nerg *et al.*, 1994, for more details) were planted in an old nursery field at the Research Nursery of the Suonenjoki Research Station, Finnish Forest Research Institute (62°39′N, 27°03′E, 130 m.a.s.l.) in 1993 (Table 1). The soil in the nursery bed was a mixture of fine sand and peat (<3% w/w). Two hundred seedlings from each origin were planted with a spacing of 1.0×0.5 m between the seedlings. The saplings were 8-year-old during the experimental growing season in 1998.

Samples from 16 saplings from each provenance were taken at 2–3 week intervals between 3 August and 30 November 1998. On each sampling occasion, shoots of lateral branches grown during the current year were sampled for impedance analysis of stems (termed 'non-frost exposed sample'), for determination of dry matter content, and for assessment of FH by means of controlled freezing tests ('frost exposed sample'). After sampling, the needles were immediately separated from the shoots in the laboratory. In November, owing to the below zero outside air temperatures, the shoots were first kept at 5 °C for 2 h prior to sample preparation in the laboratory.

Frost hardiness by controlled freezing test

The FH of the stems and needles was determined by controlled freezing tests. For the tests, 8 stems (16 in the last two tests) and 32 needles from each origin and each test temperature were prepared in plastic bags. Distilled water was sprayed into the bags to avoid excessive supercooling. In each test 6–7 freezing temperatures and a control temperature of +3 °C were used to determine the critical temperature range for FH. The freezing temperatures used were between 0 °C and −130 °C according to the predicted hardiness level. Both the initial and end temperature of the exposure was 3 °C and the rate of cooling and warming was 5 °C h^{-1}. The samples were kept at the target temperature for approximately 4 h. Immediately after the exposure the degree of frost damage in the stems and needles was quantified by the electrical impedance analysis and the electrolyte leakage method, respectively (Flint *et al.*, 1967; Repo *et al.*, 1994; Ryyppö *et al.*, 1998). The total numbers of stems and needles used for FH determination by controlled freezing tests were 2 800 and 11 500, respectively.

The FH of the frost exposed stems was determined as the extracellular resistance of each specimen obtained by means of

Table 1. *Latitude, longitude and mean temperature sum ($\Sigma T_{+5\,°C}$ as degree days) of the seed origins used in the study (mean of 30 years) (Nerg* et al., *1994)*

Group	Origin	Latitude (N)	Longitude (E)	$\Sigma T_{+5\,°C}$, d.d.
Southern	Saarenmaa	58°22′	22°51′	1450
	Tenhola	60°03′	23°04′	1300
Intermediate	Korpilahti	62°00′	25°28′	1100
	Kinnula	63°32′	25°03′	1000
Northern	Suomussalmi	65°10′	29°05′	800
	Muonio	67°56′	23°34′	700

Note: The temperature sum (in degree days) is calculated as the sum of Celsius degrees on days, starting from the beginning of year, when the daily mean temperature is above +5 °C.

electrical impedance analysis (Repo *et al.*, 1994). Immediately after the frost exposure and thawing, a 15 mm section was cut from the central portion of the stem. The section was placed directly in contact with the electrode pastes of the Ag/AgCl-cell (the type of electrodes RC1, WPI Ltd., Sarasota, U.S.A.) to measure an impedance spectrum at 42 frequencies between 80 Hz and 1 MHz (HP 4284 A) (Repo, 1994). The input voltage level of the sine signal was 100 mV (rms). The measurement of each sample took 30 s.

The distributed circuit element model (see below) was used to calculate the extracellular resistance according to the impedance spectra (Repo *et al.*, 1994). The EIS parameters of the equivalent circuit were estimated with an automated Complex Non-linear Least Squares (CNLS) fitting program (T Repo) which uses LEVM v6.0 (JR Macdonald, Department of Physics and Astronomy, University of North Carolina, Chapel Hill, NC). The FH was estimated as the inflection point of the logistic sigmoid function (equation 1).

$$y = \frac{A}{1 + e^{B \cdot (C - x)}} + D \quad (1)$$

where y and x refer to the specific extracellular resistance (unit Ωm) and the exposure temperature, respectively, A and D define asymptotes of the function, and B is the slope at the inflection point C. The specific resistance was obtained by normalization of the resistance in respect of the cross-sectional area and the length of the sample.

The FH of needles was assessed by electrolyte leakage method (Flint *et al.*, 1967; Sutinen *et al.*, 1992). After freezing, 32 needles from each provenance and each testing temperature were randomly selected. Ten millimetre pieces were cut from the middle of the needles, rinsed with distilled water and placed in test tubes (eight samples per tube) as four replicates. Six millilitres of distilled water was added to each test tube. The tubes were shaken at room temperature for 24 h before the measurement of the first conductivity (L_1). Then the samples were heat-killed at 92 °C for 20 min and shaken for another 24 h before the measurement of the second conductivity (L_2). The relative electrolyte leakage (REL) was calculated as:

$$REL = \frac{L_1}{L_2} 100 \quad (2)$$

The FH was estimated as the inflection point (parameter C) of equation 1, where y refers to the relative electrolyte leakage (REL).

Impedance analysis of non-frost exposed stems

The electrical impedance spectra of the 16 stems (one stem per sapling) from each origin at each sampling time were measured in the laboratory in the way described above immediately after sampling (a total of 8 times). The total number of non-frost exposed samples used for the EIS-analysis was 768.

The impedance spectra of stems were modelled by an equivalent circuit with two distributed circuit elements (DCE) in series with a resistor (double-DCE model) (Repo *et al.*, 1994). Both DCE-elements are composed of a constant phase element in parallel with a resistor (Fig. 1; Appendix 1) (Macdonald, 1987). The total complex impedance (Z) of the double-DCE is as shown (for derivation of this equation see Appendix 1):

$$Z = R + \frac{R_1}{1 + (i\tau_1\omega)^{\psi_1}} + \frac{R_2}{1 + (i\tau_2\omega)^{\psi_2}} \quad (3)$$

where the angular velocity $\omega = 2\pi f$ (f = frequency). In the double-DCE model, there are three resistances (R, R_1 and R_2), two relaxation times (τ_1 and τ_2) and two distribution coefficients (ψ_1 and ψ_2) of the relaxation times (for mathematical interpretation of each parameter see Fig. 1). The letter i refers to the imaginary unit. The parameters were estimated with the automated CNLS program (see above).

Since the low frequency current may not pass the cell membranes but flows in the apoplastic space the extracellular resistance (R_e) is obtained as:

$$R_e = R + R_1 + R_2 \quad (4)$$

At high frequencies the current may pass the cell membranes and accordingly flows both in the apoplastic and symplastic space, the intracellular resistance (R_i) is obtained as:

$$R_i = R\left(1 + \frac{R}{R_1 + R_2}\right) \quad (5)$$

The resistance parameters were normalized with respect to the cross-sectional area ($A_s = \pi d^2/4$, d = diameter) and the length (l) of the sample in order to obtain the specific resistances (equation 6)

$$r_x = \frac{A_s}{l} R_x \quad (6)$$

Lower case letters have been used to indicate the normalized values.

Determination of the dry matter (DM) content

The same 16 shoots from each provenance as for the 'non-frost exposed' impedance measurements were used for determination of the dry matter content at each of the eight times. After fresh weight measurements, the samples were oven-dried at 80 °C for 48 h before weighing of their dry weight. The DM content was calculated as the percentage of dry weight in relation to fresh weight.

Analysis of the data

In order to compare the differences between the provenances at different times, the original data was pooled into three groups: southern, intermediate and northern, with two provenances in each (Table 1). The significance of the monthly mean differences in FH, equivalent circuit EIS parameters and DM content between the groups was tested by means of a paired t-test.

The relation of the FH of stems and needles to the equivalent circuit EIS parameters of stems and to the DM content of stems was studied by linear and exponential models, respectively. The original data (the means of each origin at each given time) from all the six provenances over the whole study period were pooled and the linear and exponential regression models were applied (SPSS 8.0 for Windows, SPSS Inc.). Then the pooled data was split according to the assessment dates, and the correlation of FH with the parameters of the non-frost exposed samples was calculated separately for each time. For the evaluation of the reliability and accuracy of the regression models, the coefficient of determination, the confidence intervals and the residuals were examined (SPSS Inc.).

Results

Frost hardiness by controlled freezing tests

There were no significant differences in the FH between the three provenance groups in August (Table 2). Differences developed in September, when the frost hardening commenced (Fig. 2). No difference was found in the FH between the provenances in November, except between

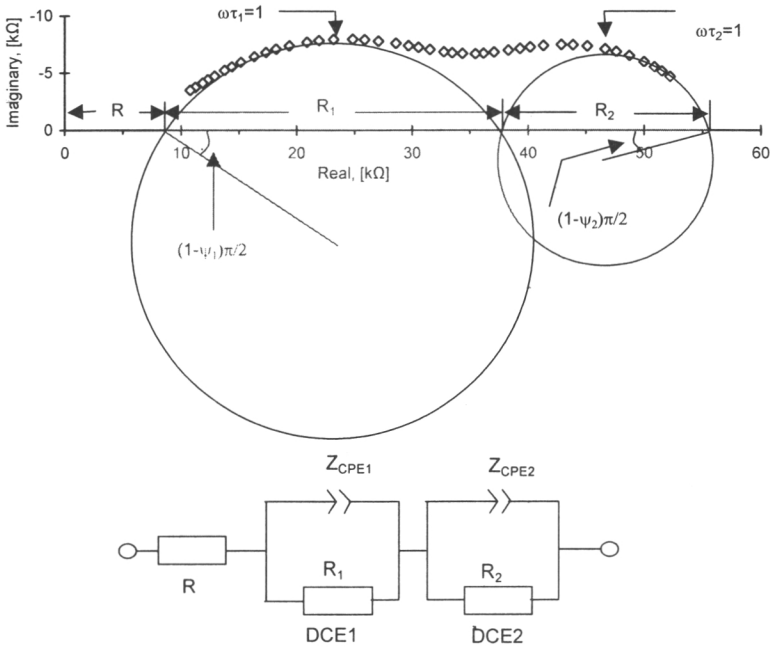


Fig. 1. Determination of the distributed model parameters (double-DCE) of an impedance spectrum of stem (◇) of Scots pine schematically. Real part of impedance on *x*-axis and imaginary part of impedance on *y*-axis (upper figure). Frequency increases from right (80 Hz) to left (1 MHz). The two circles represent the two arcs of the spectrum. DCE1 and DCE2 are distributed circuit elements composed of constant phase elements (CPE) in parallel with resistors (lower figure). Resistances (R, R_1 and R_2) of the equivalent model are obtained according to the intersections of the circles with *x*-axis. The centre of the circles is below *x*-axis→'depressed centre' defined by parameters ψ_1 and ψ_2. Relaxation times τ_1 and τ_2 are obtained from the apex of the circles. Z_{CPE1} and Z_{CPE2} are impedances of Constant Phase Elements. For more explanation see Appendix 1 and 2.

Table 2. *Comparison of frost hardiness in stems and needles between groups of provenances with time (*t*-test)*

For the test the data gathered twice per month were pooled. S–I, southern versus intermediate provenances; I–N, intermediate versus northern provenances; S–N, southern versus northern provenances. There are two origins in each provenance group.

Organ	Paired group	Significance (2-tailed) of difference			
		August	September	October	November
Stem	S–I	ns	ns	ns	ns
	I–N	ns	ns	ns	ns
	S–N	ns	**	ns	*
Needle	S–I	ns	*	*	ns
	I–N	ns	*	ns	ns
	S–N	ns	*	**	ns

Note: df = 3. Significance of the difference: ns, not significant; $*P<0.05$, and $**P<0.01$.

the stems of the southern and northern groups. The needles reached a higher level of hardiness (around −90 °C) than the stems (from −40 to −50 °C) in November.

Impedance analysis and dry matter content of non-frost exposed stems

The form of the impedance spectra of the non-frost exposed stems of Scots pine changed during the study (Fig. 3). In early August, the spectra were clearly characterized by two arcs. With frost hardening, the proportion of the low and high frequency arcs changed as the high frequency arc became more dominant.

The trend in the equivalent circuit parameters was similar for all provenances (Fig. 4). All of the resistance parameters, except r_2, increased during the study, especially from September onwards. The intracellular resistance (r_i) was 7 Ωm at the beginning of September and rose to 15 Ωm until the end of November (Fig. 4E). In September there were significant differences between the provenance groups with regard to r_i.

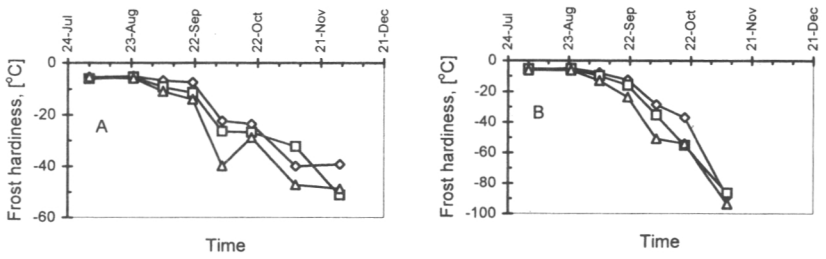


Fig. 2. The frost hardiness of stems (A) and needles (B) of Scots pine of different origins in the provenance field trial at Suonenjoki, Finland, in 1998. Each point is the mean of two origins: (◇) two southernmost, (□) two intermediate, (△) two northernmost provenances (see Table 1).

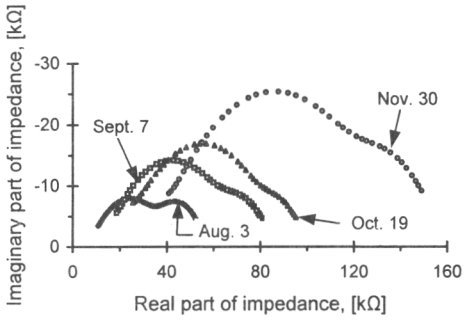


Fig. 3. Typical impedance spectra of non-frost-exposed stems of Scots pine on August 3, September 7, October 19, and November 30. The spectra are composed of 42 different frequencies ranging from 80 Hz to 1 MHz (from right to left, respectively).

The resistance r_2 decreased from 11 Ωm in August to 3 Ωm in October (Fig. 4C). The southern and northern provenances differed significantly in r_2 from the beginning of August until the end of September (Table 3).

The relaxation time τ_1 increased from 3.5 μs to 8 μs between the end of August and the end of November (i.e. the characteristic frequency f_{c1} decreased from 286 to 125 kHz). The change occurred first with the northern and last with the southern provenances (Fig. 4F). There was a time lag of approximately 1 month in $\tau_1$between the provenances; the level of 6 μs was reached on 29 September and 24 October in the southern and the northern groups, respectively. For most of the time, the differences between the provenance groups were significant (Table 3).

The relaxation time τ_2 behaved more irregularly than τ_1 (Fig. 4G). At the end of August all of the groups differed significantly with respect to τ_2 (Table 3). In mid-September, τ_2 increased from 400 μs to 550–600 μs (i.e. the characteristic frequency f_{c2} decreased from 2.5 to 1.7 kHz) concomitantly with the increase in the coefficient ψ_2 (Fig. 4I). All the provenance groups differed significantly from each other at the end of September with respect to ψ_2 (Table 3).

The DM content of the stems increased during the experimental period for all groups (Fig. 4J). There was a temporary drop in DM content on 19 October. On that date the frost hardening of the stems temporarily ceased (Fig. 2). For most of the study period the DM content of the northern and intermediate groups was significantly higher than that of the southern one (Table 3, Fig. 4J).

Comparison of the EIS parameters and the DM content of non-frost exposed samples with frost hardiness

The relaxation time τ_1 and the intracellular resistance r_i of the stems displayed the highest correlation with the FH of the stems and needles (Table 4). The linear regression model described the relation between the FH and τ_1 well (Fig. 5A, B). Some model error existed for the lowest r_i values in the linear model as the r_i increased without any clear change in FH (Fig. 5C, D). The relation between FH and DM content was non-linear (Fig. 5E, F). The DM content increased at first without hardening, which was initiated when the DM content reached a level between 32% and 35%.

In the pooled data the deviation between measured and predicted FH was occasionally high for τ_1. According to the linear model the 90% confidence intervals of FH for needles and stems were ±15 °C and ±9 °C for τ_1 and ±20 °C and ±14 °C for r_i, respectively. The predictive power of the DM content was variable; an increase of DM at low values had no effect on the FH. The FH increased with increasing DM by over 32–35%, but concomitantly the confidence intervals expanded remarkably.

At selected times in the hardening phase the correlation between the FH and the τ_1 was higher and the residuals between the predicted and measured FH lower than for the pooled data (Table 4; Fig. 6). The coefficient of determination was highest at 0.95 between the FH of the needles and the τ_1 of the stems on 21 September (Fig. 6B). Then the residuals were within ±2.0 °C. The slope of the regression line gradually decreased from 24 August to 5 October with increasing hardiness (Fig. 6A). The variation of the residuals typically increased with the increase in FH. The correlation of FH with other parameters than

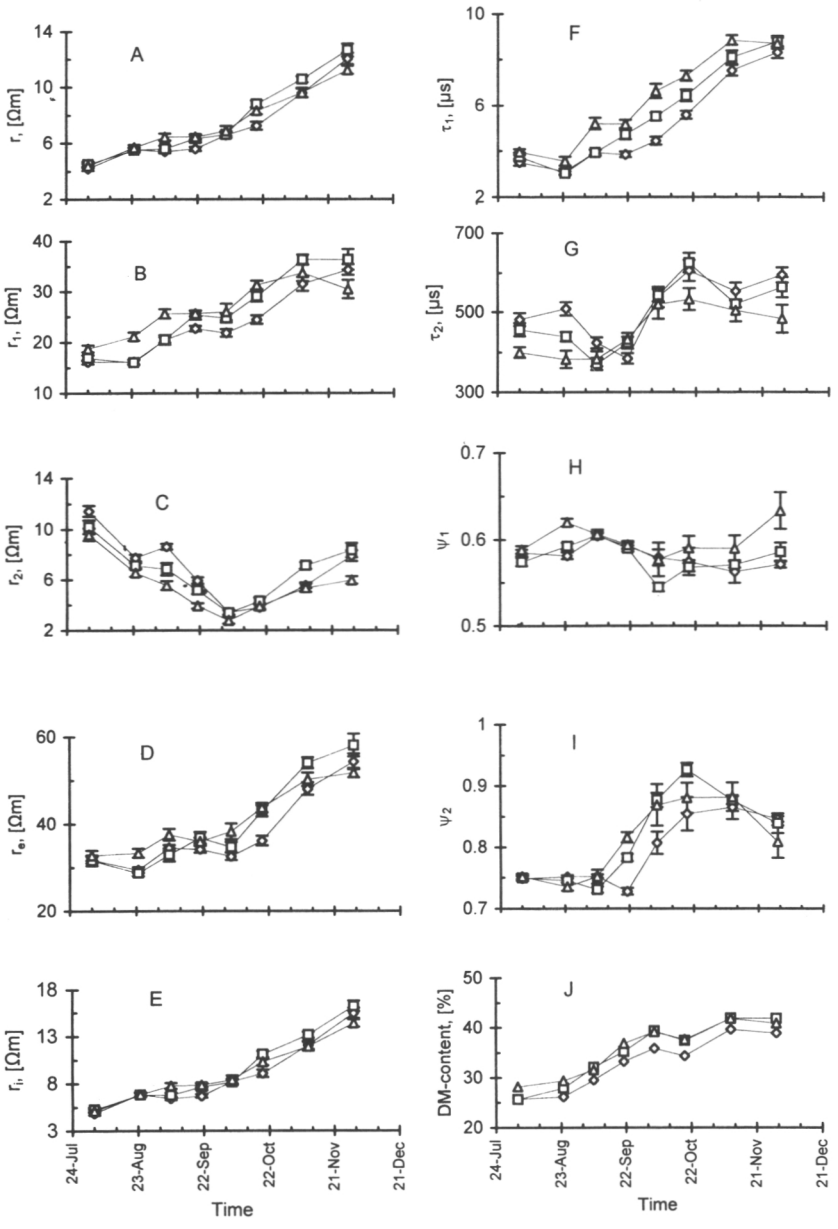


Fig. 4. The parameters for the double-DCE model of the non-frost-exposed stems of Scots pine by impedance analysis (A–I) and the dry matter content (DM) of stems (J) of southern (◇), intermediate (□) and northern (△) provenances (see Table 1) in a field trial with Scots pine. The parameters r, r_1, r_2, r_e and r_i are resistances, and ψ_1 and ψ_2 are the distribution coefficients of the relaxation times τ_1 and τ_2, respectively. Each point is the mean of two origins ($n = 32$, 16 per origin). The bars indicate standard errors.

τ_1 was temporarily high and significant, e.g. for r_2-FH_{stem} and r_2-FH_{needle} on 21 September, r_i-FH_{needle} on 7 September, ψ_2-FH_{needle} on 21 September and DM-FH_{stem} on 30 November (Table 4).

Comparison of the EIS parameters and the dry matter content

According to the non-linear regression model for stems, the dry matter content had the highest coefficient of

Table 3. *The dates when the differences between the provenance groups in the electrical impedance spectroscopic (EIS) parameters and the dry matter content (DM) of stems of Scots pine were statistically significant*

Otherwise the differences were not significant. The paired provenance groups were: (A) Southern versus intermediate. (B) Intermediate versus northern. (C) Southern versus northern.

EIS parameter	Significance (2-tailed) at different times							
	3 Aug	24 Aug	7 Sept	21 Sept	5 Oct	19 Oct	7 Nov	30 Nov
(A) Southern versus intermediate								
τ_1				**	***	*		
r_1				**		**		
r_2			***				***	
r_e						***	*	
τ_2		**	*					
r				*		*	*	
r_i				*		*		
ψ_1					**			
ψ_2			**	***				
DM content		***	**	**	***	***	*	**
(B) Intermediate versus northern								
τ_1	*	*	***		***	***		
r_1	**	***	***	***				
r_2				**			***	**
r_e		**	*		***	***		
τ_2	***	***		**				**
r			***	***		*		
r_i			***	***		*		
ψ_1		***						**
ψ_2		*		***				
DM content	***	*						
(C) Southern versus northern								
τ_1		*	***	***	***	**	***	
r_1	*	***	***			***		
r_2	*	**	***	***				**
r_e		***	*		**			*
τ_2	**	*				**		
r			**				*	*
r_i			**					*
ψ_1	*	***						*
ψ_2				**				
DM content	***	***	*	**	***	***	**	*

Significance of difference (df = 31): $*P<0.05$; $**P<0.01$; $***P<0.001$.

determination with the τ_1 and r_1 which both increased with an increase in the DM content (Fig. 7). There was a minor change in the parameters when the DM content increased from 25% to 35%. Thereafter τ_1 and r_1 increased faster with a further increase in the DM content above 40%.

Discussion

The electrical impedance spectra of Scots pine saplings changed during the study period in autumn. The changes were mainly similar to those found previously (Repo *et al.*, 1994, 1995, 1997), i.e. the magnitude of the real and the imaginary levels as well as the proportion of the high and low frequency arc changed. The CNLS analysis of the spectra modelled with the double-DCE showed that, all of the impedance parameters of the non-frost-exposed stems changed during the study period between early August and late November. Typically, there was first a latent period in August and early September when the parameters remained fairly constant and no frost hardening was found. Then the parameters increased concomitantly with frost hardening. The resistance r_2 formed an exception to that trend, since it already decreased in August without any indication of frost hardening or increase in DM content, and continued to do so until early October.

The relaxation time τ_1 had the highest correlation with FH compared with the other equivalent circuit parameters or the DM content. Thus the τ_1 seemed to predict the FH without a controlled freezing test. On some occasions the deviation between the predicted and measured FH was high, however, e.g. ranging up to 25 °C for τ_1 between 4 μs and 6 μs (Fig. 5A). Due to the large deviation between the measured and predicted FH of the samples covering the temperature range from −5 °C to −90 °C, none of the parameters were accurate enough for applications.

Table 4. *The coefficient of determination* (R^2) *of the linear regression model for the comparison of the electrical impedance spectroscopic (EIS) parameter and dry matter (DM) content of stems (non-frost exposed samples) with frost hardiness of stems and needles* (FH_{stem} *and* FH_{needle}, *respectively) (frost exposed samples) at dates when the correlation was significant*

(A) EIS parameter of stems versus FH_{stem}. (B) EIS parameter of stems versus FH_{needle}. (C) DM content of stem versus FH_{stem} and FH_{needle}. The EIS parameters were obtained by the double-DCE model. The linear regression was calculated using the pooled data of all provenances and test dates ($n=48$) and at different dates separately ($n=6$).

	Pooled data	Coefficient of determination at different dates							
		3 Aug	24 Aug	7 Sept	21 Sept	5 Oct	19 Oct	9 Nov	30 Nov
(A) EIS parameter of stem versus FH_{stem}									
τ_1–FH_{stem}	0.87***					0.88***			
r_i–FH_{stem}	0.74***							0.71*	
r–FH_{stem}	0.74***								
r_e–FH_{stem}	0.69***					0.70*			
r_1–FH_{stem}	0.62***								
ψ_2–FH_{stem}	0.49***				0.76*				0.75*
τ_2–FH_{stem}	0.33***		0.76*						
r_2–FH_{stem}			0.73*	0.75*	0.83*				0.75*
ψ_1–FH_{stem}			0.69*						0.69*
(B) EIS parameter of stems versus FH_{needle}									
τ_1–FH_{needle}	0.89***			0.84*	0.95**	0.90**			
r_i–FH_{needle}	0.85***			0.83*					
r–FH_{needle}	0.85***			0.79*					
r_e–FH_{needle}	0.76***		0.77*				0.75*		
r_1–FH_{needle}	0.73***		0.78*	0.79*					
ψ_2–FH_{needle}	0.69***				0.90**				
τ_2–FH_{needle}	0.38***				0.67*				
ψ_1–FH_{needle}	0.13*								
r_2–FH_{needle}					0.88**				
(C) DM content of stem versus FH_{stem} and FH_{needle}									
DM_{stem}–FH_{stem}	0.75***								0.87**
DM_{stem}–FH_{needle}	0.71***	0.73*	0.67*						

Statistical significance: $*P<0.05$; $**P<0.01$; $***P<0.001$.

At selected times during the hardening period the coefficient of determination between the FH and the relaxation time τ_1 was high, and the deviation between the predicted and measured FH was small. These assessments were located in the initial phase of hardening which is the most interesting phase in several applications. During that time the differences between the provenances were the most obvious, i.e. the FH of needles of different provenances were between −10 °C and −25 °C. Thus, in that phase of hardening it was possible to grade the provenances according to their hardening pattern without a controlled freezing test.

Interpretation of the EIS parameters

In mathematical terms, the τ_1 is obtained from the apex of the high frequency arc of the impedance spectrum (Fig. 1). Using the normalized values for r_1 and c_1 we get for the relaxation time $\tau_1 = r_1^{1\,\psi_1} c_1$, with the capacitance c_1 representing some interface in the tissue. Then the c_1 remained fairly constant during the study period, ranging from 10–40 nF m^{-1} (data not shown), whereas the parameters τ_1 and r_1 were linearly correlated. This suggests that the change in the τ_1 is due to the r_1 rather than the c_1, and thus the capacitance c_1 would not change during frost hardening. The increase in τ_1 is then caused by the change in the ion mobility in a cellular compartment represented by r_1.

A biological interpretation of the τ_1 and the derived parameters r_1 and c_1 is unknown. It has been proposed that altered cell membrane properties affect the relaxation time of the leaves and stems of olive trees (*Olea europaea* L.) (Mancuso and Rinaldelli, 1996) and accordingly r_1 and/or c_1. If a general number of 1 μF cm^{-2} is used for the cell membrane capacitance and a cell membrane thickness of 10 nm is assumed, the specific capacitance of 0.1 nF m^{-1} is obtained for the cell membrane. This number is about 100 times smaller than that calculated from this study's data.

The relaxation time τ_1 and the resistance r_1 increased with an increase in the dry matter content. Thus, the water content may partially explain the behaviour of τ_1 and r_1. Change or more specifically redistribution of water between symplast and apoplast may partially explain the decrease in the τ_1 in case of freezing tests too (Repo *et al.*, 1994). The net water content of the

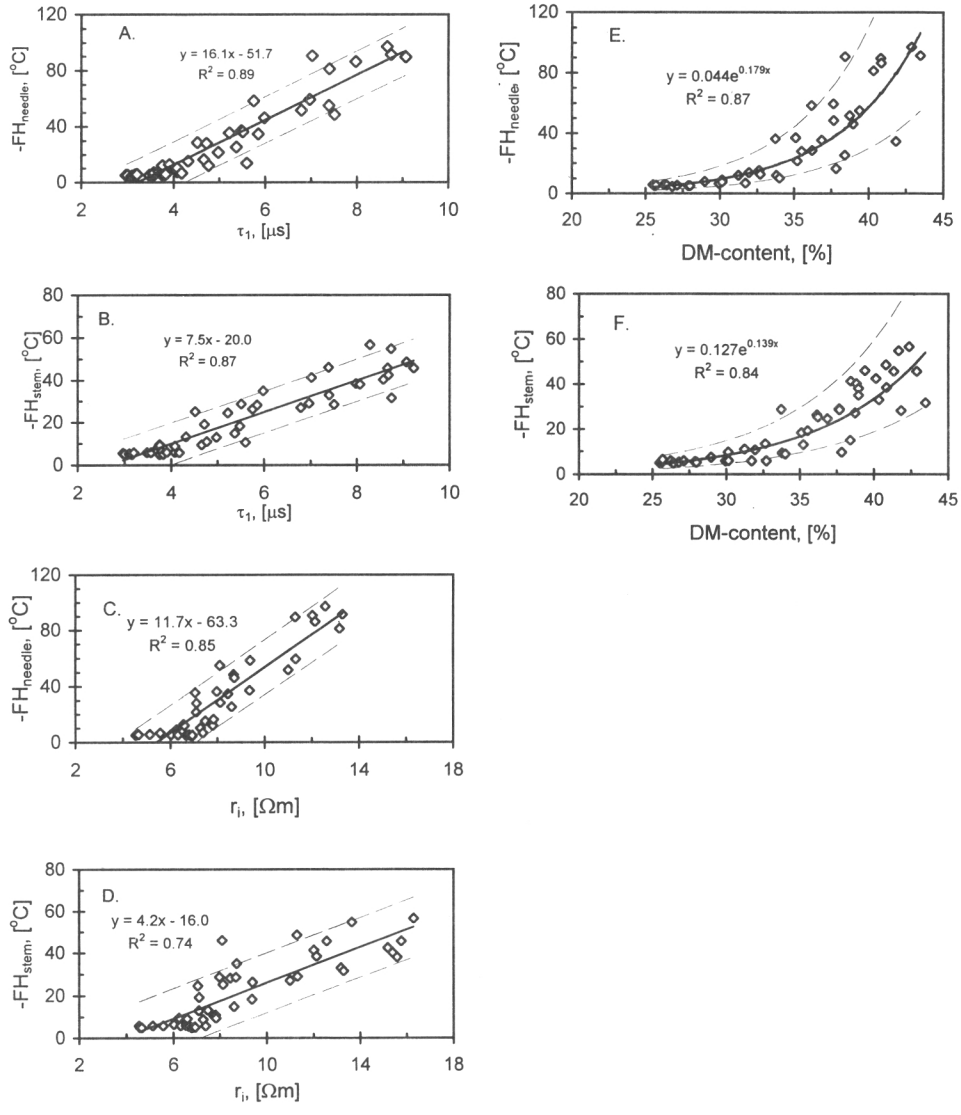


Fig. 5. The relationship of the frost hardiness of needles and stems with the relaxation time τ_1 (A, B), intracellular resistance r_i (C, D), and the dry matter (DM) content (E, F) of non-frost-exposed stems of Scots pine. The data from six provenances and different times from August to November have been pooled. Each point represents the mean of one origin at one time: τ_1, r_i and the DM are the means of 16 stems, and the respective frost hardiness is obtained by means of controlled freezing tests (impedance analysis and electrolyte leakage for stems and needles, respectively). The best fit function, the coefficient of determination (R^2) and the 90% confidence intervals are indicated.

tissues does not change there, but there is a drift of water towards apoplastic ice by exosmosis. This together with damage in cell membranes and consequent ion leakage from the symplast to the apoplast (Palta and Weiss, 1993; Ryyppö *et al.*, 1998) leads to decrease in τ_1. This may, in turn, contribute to a decrease in the r_1 due to an increase in the charge-carrying ion content inside or outside the cell.

The dry matter content of the stems ranged from 25% to 45%, i.e. the moisture content (MC) from 75% to 55%, respectively. Thus the MC was well above the fibre saturation point (FSP) of 30% in woody plants. Above the FSP, the low frequency impedance should not depend much on the moisture content (Tattar *et al.*, 1972; Glerum, 1980; Pukacki, 1982; Kucera, 1986), but this effect may not totally absent, as these data reveal. This

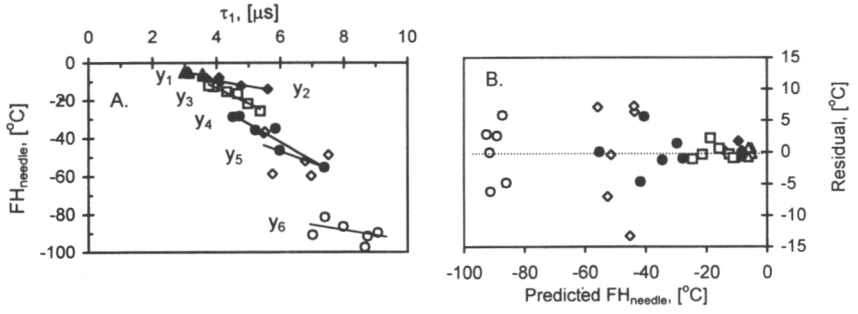


Fig. 6. (A) The relationship between the frost hardiness of needles and the relaxation time τ_1 of stems of Scots pine at different times: 24 August (y_1, △), 7 September (y_2, ◆), 21 September (y_3, □), 5 October (y_4, ●), 19 October (y_5, ◇) and 9 November (y_6, ○). Each point of the τ_1 is the mean of 16 stems. The FH of the needles is assessed by means of electrolyte leakage method. The best fit regression lines are indicated. (B) The residuals between the measured and predicted FH for the linear regressions as a function of predicted FH at different times (for symbols see (A)).

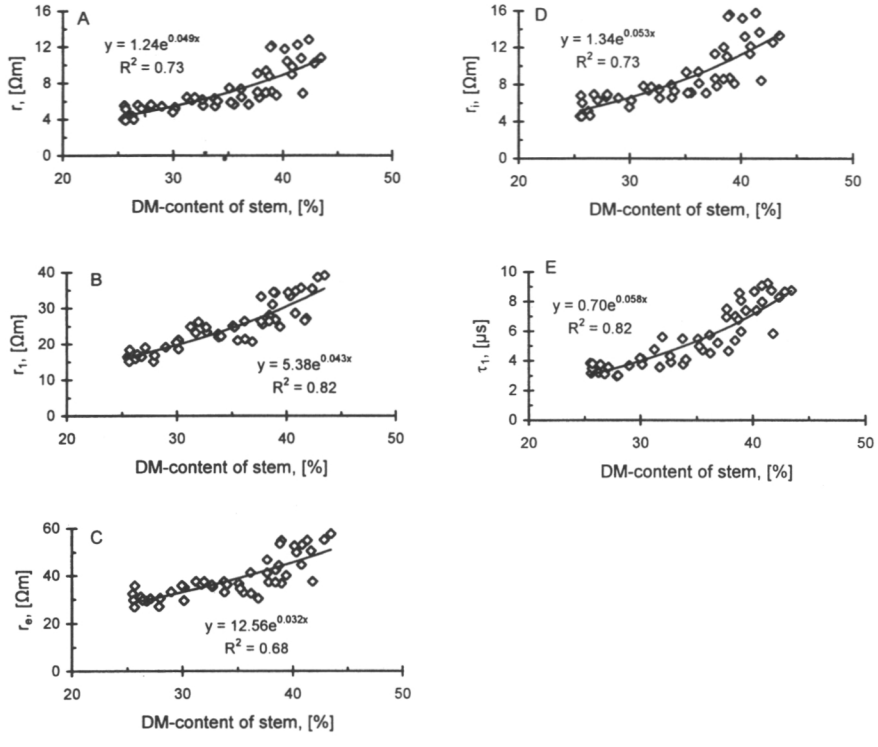


Fig. 7. The relationship between the dry matter content (DM) and the equivalent circuit parameters of the double-DCE model of the non-frost-exposed stems of Scots pine. The data from all six provenances have been pooled. Each point is the mean of 16 stems. The best fit exponential function with the coefficient of determination (R^2) is indicated.

study's data also include the acclimation period and the concomitant changes in the cellular properties, for example, cell wall thickness and rigidity, the size of the apoplastic space and membrane fluidity and symplastic compartmentalization. Those changes may partly explain the increase in certain EIS parameters which took place coincidentally with the decrease in the water content.

Prior to the hardening phase in August, when the diameter growth of the stems was ceasing, the relaxation time τ_2 of the southern and intermediate provenances was higher than that of the northern ones. The resistance r_2 decreased from August to September and the decrease occurred later in the southern than in the northern origins. The parameters τ_2 and r_2 were not correlated

(data not shown). According to the relation $\tau_2 = r_2^{1/\psi_2} c_2$, the capacitance c_2 decreased exponentially from 400 to 10 μF m^{-1} with an increase of r_2 from 2 to 15 Ωm, i.e. the c_2 increased prior to the frost hardening. The capacitance values of the c_2 were 1000 to 10 000 times higher than the values of the c_1 and more than 1×10^5 times higher than the cell membrane capacitance. Judging from the timing of the change in the r_2 and τ_2 it can be assumed that r_2 and τ_2 are connected with cellular differentiation and lignification.

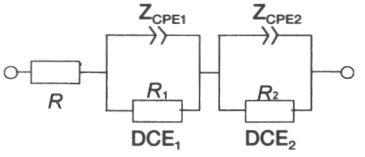
The intracellular resistance r_i increased with frost hardening as was found earlier in Scots pine (Repo *et al.*, 1995), alfalfa (*Medicago stativa* L.), birdsfoot trefoil (*Lotus corniculatus* L.) (Stout 1988*a*, *b*) and willow (*Salix viminalis*) (Repo *et al.*, 1997). In the pooled data the coefficient of determination of FH for r_i was less than that for τ_1, however, but it was occasionally as high as 0.83 (Table 4B). The increased concentration of the intracellular sap and the impaired intracellular ion mobility due to 'frictional effect' (Pauly and Schwan, 1966) may explain the increase in the r_i with frost hardening.

The DM content of stem started to increase before frost hardening. After the DM content of stems had increased to over 30%, the FH increased steeply in accordance with the previous studies (Junttila and Kaurin, 1990; Toivonen *et al.*, 1991; Sutinen, 1992; Repo *et al.*, 1997). The decrease in the water content is probably one of the first reactions at the moment of growth cessation and in reaction to physiological and structural changes in cells preceding cold acclimation. Significant differences were found in the DM content between provenances during frost hardening, but the DM content as such was not a reliable method for the determination of FH without a controlled freezing test. The DM content and FH of stems decreased temporarily on 19 October. This decrease can be explained by a fairly warm and rainy period just before that sampling date.

In conclusion, impedance spectroscopic analysis is a useful method for studying cold acclimation. The method is fast, and with proper planning over 300 samples can be measured in 1 d which is an advantage with regard to most other methods. The relaxation time of the non-frost exposed stems of Scots pine showed a high correlation with their frost hardiness. With proper timing of the measurement, the accuracy of impedance analysis may be high enough for forestry applications in nurseries, in seed orchards and in provenance trials for grading genotypes for the timing of frost hardening.

Appendix 1

The double-DCE model is composed of two distributed elements (DCE_1 and DCE_2) in series with a resistor (R). Both DCEs are composed of a parallel arrangement of a resistor R_1 and R_2 and a constant phase element Z_{CPE1} and Z_{CPE2}, respectively.



The impedance of the Constant Phase Elements are (Macdonald 1987, 90–95):

$$Z_{CPE_1} = \frac{1}{(j\omega C_1)^{\psi_1}} \quad \text{and} \quad Z_{CPE_2} = \frac{1}{(j\omega C_2)^{\psi_2}}$$

For the impedance of the DCEs we get:

$$\frac{1}{Z_{DCE_1}} = \frac{1}{R_1} + \frac{1}{Z_{CPE_1}} \quad \text{and} \quad \frac{1}{Z_{DCE_2}} = \frac{1}{R_2} + \frac{1}{Z_{CPE_2}}$$

Then

$$Z_{DCE_1} = \frac{R_1}{1 + R_1(j\omega C_1)^{\psi_1}} = \frac{R_1}{1 + (j\omega C_1 R_1^{1/\psi_1})^{\psi_1}}$$

and $$Z_{DCE_2} = \frac{R_2}{1 + R_2(j\omega C_2)^{\psi_2}} = \frac{R_2}{1 + (j\omega C_2 R_2^{1/\psi_2})^{\psi_2}}$$

When we define $\tau_1 = C_1 R_1^{1/\psi_1}$ and $\tau_2 = C_2 R_2^{1/\psi_2}$

We get

$$Z_{DCE_1} = \frac{R_1}{1 + (j\omega\tau_1)^{\psi_1}} \quad \text{and} \quad Z_{DCE_2} = \frac{R_2}{1 + (j\omega\tau_2)^{\psi_2}}$$

For the total impedance of the double-DCE model we get:

$$Z = R + Z_{DCE_1} + Z_{DCE_2} = R + \frac{R_1}{1 + (j\omega\tau_1)^{\psi_1}} + \frac{R_2}{1 + (j\omega\tau_2)^{\psi_2}}$$

Appendix 2

List of symbols and abbreviations:

Symbol	Explanation	Unit
A	coefficient to define the asymptote of the sigmoid function	% or Ωm
B	slope of the sigmoid function at the inflection point	% $°C^{-1}$ or Ωm $°C^{-1}$
C	inflection point of the sigmoid function	°C
D	coefficient to define the asymptote of the sigmoid function	% or Ωm
L_1	conductivity	μS cm^{-1}
L_2	conductivity	μS cm^{-1}
REL	relative electrolyte leakage	%
FH	frost hardiness	°C
DM	dry matter content: (dry/fresh) × 100	%
CNLS	Complex Non-linear Least Squares	
DCE	Distributed Circuit Element model	
Z	complex impedance	Ω

Z_{CPE1}	impedance of the Constant Phase Element 1	Ω
Z_{CPE2}	impedance of the Constant Phase Element 2	Ω
i	imaginary unit	
$\omega = 2\pi f$	angular velocity	$rads^{-1}$
f	frequency	Hz
R_x	resistance	Ω
r_x	specific resistance	Ωm
R, R_1, R_2	resistances in the double-DCE model	Ω
R_e	extracellular resistance	Ω
R_i	intracellular resistance	Ω
r, r_1, r_2	specific resistances in the double-DCE	Ωm
r_e	specific extracellular resistance	Ωm
r_i	specific intracellular resistance	Ωm
τ_1	relaxation time	s
τ_2	relaxation time	s
f_{c1}	characteristic frequency $= 1/\tau_1$	Hz
f_{c2}	characteristic frequency $= 1/\tau_2$	Hz
ψ_1	distribution coefficient of τ_1	
ψ_2	distribution coefficient of τ_2	
c_1	specific capacitance	Fm^{-1}
c_2	specific capacitance	Fm^{-1}
d	diameter	m
A_s	cross-sectional area	m^2
l	length of the sample	m
R^2	coefficient of determination	

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III

THE ELECTRICAL IMPEDANCE SPECTROSCOPY OF SCOTS PINE NEEDLES DURING COLD ACCLIMATION

Gang Zhang[1, *], Aija Ryyppö[1], and Tapani Repo[2]

[1] Faculty of Forestry, University of Joensuu, P.O. Box 111, FIN-80101 Joensuu, Finland.
[2] The Finnish Forest Research Institute, Joensuu Research Centre, P.O. Box 68, FIN-80101 Joensuu, Finland.

Author to whom [2)] all correspondence should be directed: Fax: +358 13 2514567. E-mail: tapani.repo@metla.fi

ABSTRACT

The electrical impedance spectroscopy (EIS) was applied to current-year needles of Scots pine (*Pinus sylvestris* L.) in an eight-years provenance field trial in central Finland during cold acclimation. The EIS analysis of the needles using a Model-A equivalent circuit (which takes account of the presence of air spaces within the needles; the letter A stands for air space) indicated a sequence of events in the needles during their cold acclimation. Some of the EIS-parameters referred to maturation phenomena occurring during the prehardening phase at the end of the growing season, and some parameters displayed a clear coincidence with the frost hardening itself. Significant differences between provenances were found in several of the Model-A parameters. Extracellular resistance (r_e) and β-coefficient (a factor controlling the skewness of the spectrum and the impedance locus centre depression) decreased in all provenances in the prehardening phase in August and until mid-September. In the same phase, both the intracellular resistance (r_i) and the cell membrane time constant (τ_m) first increased and then decreased. According to τ_m, r_e and β there was a clear gradation between provenances in the prehardening phase. From the end of September significant differences were found in the intracellular resistance between provenances, corresponding with the differences in their hardening pattern. The dry matter (DM) content of needles increased during the study period but no clear differences were found between the provenances.

Key words: dry matter content, frost hardiness, impedance spectroscopy, *Pinus sylvestris*.

* Former address: Faculty of Horticulture, Agricultural University of Hebei, Baoding, Hebei. 071001. P. R. China.

INTRODUCTION

Cold acclimation in plants consists of several physiological, biochemical, biophysical and anatomical changes in cells and tissues (Weiser 1970, Levitt 1980, Steponkus 1984). Changes in the composition of cell membranes, cytoplasm and vacuole are central during this process. Most of the methods used to study cellular changes during cold acclimation are time consuming and may require a considerable amount of material. Thus they may not be useful for practical applications. Accordingly, there is a considerable challenge to develop a simpler and faster method of studying the cold acclimation of plants.

One of the methods that has been used to study cold acclimation in plants is electrical impedance spectroscopy (EIS) (Repo et al. 1995, 1997, 2000a). EIS has also been used previously to characterise the properties of biological materials (Zhang and Willison 1991, Repo and Zhang 1993), and it may provide information about the structural changes in plant cells and tissues during their annual cycle (Mancuso 1998). Intracellular resistance and relaxation time in stems of willow (*Salix viminalis* L.) and Scots pine (*Pinus sylvestris* L.) have displayed a high correlation with frost hardiness (FH) (Repo et al. 1995, 1997, 2000a). We may, therefore, also expect changes in the electrical properties of needles during cold acclimation. Thus far, there exist, however, no comprehensive EIS-studies of needles that have been concerned with discovering the nature of the changes in their electrical properties in relation to their cold acclimation.

The dry matter (DM) content on woody plant shoots increases with cold acclimation (Junttila and Kaurin 1990, Toivonen et al. 1991, Repo et al. 1997, 2000a). As a result of its simplicity, the method used appears to have practical applicability, e.g., in nurseries. The predictive power of the DM content for assessing the FH is variable, however. In previous studies, the DM content of plant tissues started to increase before the frost hardening had started. The increase in the DM content proceeded faster than the FH increased in the first stage of cold acclimation, and the change in the DM content also ended earlier than the frost hardening ceased (Sutinen 1992, Repo et al. 1997, 2000a). In the case of Scots pine stems, the FH increased with the increasing DM content by over 32-35%, but concomitantly the confidence intervals expanded remarkably (Repo et al. 2000a).

Our aim was to study the electrical properties of Scots pine needles of different provenances by means of EIS during the summer and autumn cold acclimation phase and

to compare the changes in EIS properties with their DM content and their FH. We hypothesised that, as a result of physiological and anatomical changes in the needles during cold acclimation, their EIS properties would change and that the provenances would differ in their EIS properties. Accordingly, the cold acclimation process of the needles may be followed by means of EIS.

MATERIALS AND METHODS

Plant material

The study was based on a field provenance trial involving six different Scots pine (*Pinus sylvestris* L.) origins. The seeds for the trial were collected from six open-pollinated natural stands over an area ranging from Estonia to northern Finland. The bareroot 2+0 seedlings (see Nerg et al. 1994 for more details) were planted in 1993 in an old nursery field at the Research Nursery of the Suonenjoki Research Station run by the Finnish Forest Research Institute (62°39'N, 27°03'E, 130 m a.s.l.) (Table 1). The nursery soil was a mixture of fine sand and peat (<3% w/w). Two hundred seedlings from each provenance were planted with a spacing of 1.0m x 0.5m between the seedlings. The saplings reached their eighth growing season in 1998.

Table 1. Latitude and longitude of the Scots pine seed origins used in the study (Nerg et al. 1994).

Group	Origin	Latitude (N)	Longitude (N)
1. Southern	Saarenmaa	58°22'	22°51'
	Tenhola	60°03'	23°04'
2. Intermediate	Korpilahti	62°00'	25°28'
	Kinnula	63°32'	25°03'
3. Northern	Suomussalmi	65°10'	29°05'
	Muonio	67°56'	23°34'

Sixteen sampling trees were chosen from each provenance according to the mean height of the provenance. The measurements of electrical impedance, frost hardiness and

dry matter content of needles started on 3 August and continued until 4 December 1998. The shoot samples were taken from the first lateral branches at 2-3 week intervals. After sampling, the needles were immediately separated from the shoots. Owing to the low air temperature in November, the samples were first thawed at 5°C for 2 hours prior to further sample preparation. The temperature record over the study period has been presented previously (Repo et al. 2000b).

EIS of the non-frost-exposed needles

Sixteen randomly-selected needles from 16 sampling shoots taken from each provenance were used for the impedance spectroscopic measurements. In order to avoid drying, the needles were kept in plastic bags at a room temperature of 24°C before the measurements were made. *These needles are termed non-frost-exposed samples, i.e., the samples were not artificially exposed to frost by controlled freezing tests.*

The electrical impedance spectra of the 16 needles from each provenance at each sampling time were measured in the laboratory immediately after sampling (a total of 8 times). The total number of non-frost-exposed needles used for these studies was 768. A 15-mm section was cut from the middle of each needle. The impedance spectra were measured in the manner described by Repo (1994), except that the Ag/AgCl-electrodes (RC 1, WPI Ltd., Sarasota, U.S.A) were set in direct contact using electrode paste, and the sample was placed between the pastes. The impedance spectrum was measured at 46 frequencies between 20 Hz and 1MHz (HP 4284 A). The input voltage level of the sine signal was 100mV (rms).

The impedance spectra of the needles were modelled by means of an equivalent circuit Model-A (which takes account of the presence of air spaces within the needles; the letter A stands for air space) (Zhang et al. 1995). The impedance of the Model-A is represented by the equation:

[1] $$Z_{Model-A} = R_\infty + \frac{(R_o - R_\infty) \bullet (1+\beta)}{1+\beta \bullet (1+j \bullet \omega \bullet \tau_m)^{0.5}}$$

where R_∞ and R_0 are resistances at very high and very low frequencies, respectively. The membrane time constant is shown as $\tau_m = R_3 \bullet C_m$, where R_3 and C_m are the resistance and the capacitance of the cell membrane, respectively. The coefficient β is a factor controlling the skewness of the spectrum and the impedance locus centre depression (for a biological

interpretation of β, see Zhang et al. 1995). The complex number operator is $j=\sqrt{-1}$, and $\omega=2\bullet\pi\bullet f$ is the angular velocity (f = alternating current frequency).

The low frequency resistance R_0 corresponds to the extracellular resistance R_e. The intracellular resistance was calculated as:

[2] $$R_i = R_\infty \bullet \frac{R_o}{(R_o - R_\infty)}$$

The specific resistances r_x were calculated as:

[3] $$r_x = R_x \bullet \frac{A_c}{l}$$

where R_x is the estimated resistance and A_c is the cross-sectional area of the needle. The cross-section was assumed to be semi-elliptical. Accordingly, the area is $A_c=\frac{\pi \bullet a \bullet b}{4}$, where a is the thickness of the needle and b the width of the needle. The letter l indicates the length of the sample (l=15 mm).

The parameters of Model-A were estimated using an automated complex non-linear least squares (CNLS) fitting program (T. Repo), which uses LEVM v 6.0 (obtained from J. R. Macdonald, Department of Physics and Astronomy, University of North Carolina, Chapel Hill, NC).

FH of the needles assessed by controlled freezing tests

On each of the eight sampling occasions, 32 needles taken from each provenance were placed in plastic bags for each exposure temperature (6-7 freezing temperatures and control at +3°C). Distilled water was sprayed into the bags to avoid excessive supercooling. The temperature range used for the freezing tests was from 0°C to –130°C. The tests were conducted in air-cooled chambers (an external alcohol-circulating system: Lauda RUK90 Ultra-Kryomat together with a Lauda digital programmer R410 and PM351 (MGM Lauda, Germany)), except that an N_2-gas cooled chamber (GCC-30, Carbolite, UK with Taylor-Whatron XL-180 liquid N_2-tank) was used for the lowest test temperature. The test

temperatures were decided according to previous FH test results in order to cover the assumed critical temperature range for freezing damage. The programmed initial and end temperature of each exposure was 3°C and the samples were kept at the target temperature for 4 hours. The rate of cooling and warming was 5°C/h.

The FH of the needles was estimated by using the electrolyte leakage method (Flint et al. 1967, Hurme et al. 1997, Repo et al. 2000a,b). After the exposure, 10mm cuttings were taken from the middle of the needles. The samples were rinsed with distilled water and placed in test tubes (eight samples per tube and four replicates per temperature). Six milliliters of distilled water were added to each test tube and the tubes were shaken at room temperature for 24-h before the first conductivity measurement (L_1). Then the samples were heat-killed at 92°C for 20 min and shaken for another 24-h before the second measurement of conductivity (L_2). The relative electrolyte leakage (REL) was calculated as:

[4] $$REL = \frac{L_1}{L_2} \bullet 100$$

The FH was estimated with the response of REL to the exposure temperature, i.e. as the inflection point (parameter C) of Eq. [5] (Repo and Lappi 1989).

[5] $$y = \frac{A}{1 + e^{B \bullet ((C - x)}} + D$$

where y and x refer to the relative electrolyte leakage (REL) and the exposure temperature, respectively, A and D define asymptotes of the function, and B is the slope at the inflection point C.

The total number of needles used in the electrolyte leakage tests was 11500.

Determination of the DM content

The fresh and dry weights were measured for the same needle samples (16/provenance) as those used in the impedance analysis. After the determination of the impedance spectra, the fresh weight of the pooled samples was measured and the samples were dried at 80°C for 48-h. The DM content was calculated as a percentage of the fresh weight.

Statistical analysis

This study is based on a comparison of the equivalent circuit EIS-parameters and DM content of non-frost-exposed needles with the FH of needles determined by means of controlled freezing tests. First, the 6 provenances were divided into three groups, i.e., as southern, northern and intermediate provenances (Table 1) and the time course of the group mean values was examined. The significance of the mean difference in the FH and DM content between the groups was tested using Analysis of Variance (SPSS 8.0 for Windows, SPSS Inc.). The significance of the mean difference in the EIS parameters between the groups was tested by means of the Univariate Analysis of Variance (SPSS 8.0 for Windows, SPSS Inc.). Second, the mean values for the DM content and the different EIS-parameters were calculated for each provenance on each assessment date. The data collected on the various dates were pooled and comparisons were made between the different variables, including their FH. Regression analysis was then applied to the data. In order to evaluate the reliability of the regressions, the coefficients of determination (R^2) were subjected to examination (Microsoft Excel).

RESULTS

EIS analysis of needles

The shape of the impedance spectra of the non-frost-exposed needles of Scots pine changed during the study period (Fig.1). In early-August the spectra were characterized by two strongly overlapping arcs, while from the beginning of September onwards only one arc was present. The magnitude of the real and imaginary parts was about the same at low frequencies from August until early-September and also from mid-October until late-November.

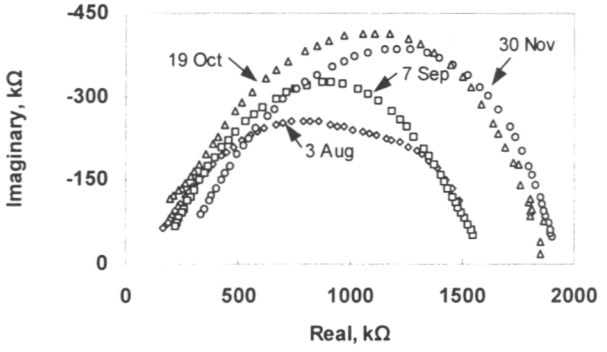

Figure 1. Impedance spectra of non-frost-exposed needles (the samples were not artificially exposed to frost) of Scots pine on: 3 August (◊), 7 September (□), 19 October (Δ), and 30 November (o). The spectra are the pooled data of six provenances and are composed of 46 different frequencies ranging from 20 Hz to 1 MHz (right to left, respectively).

All the Model-A parameters changed during the study period. There were different phases in this alteration. The first phase lasted from August until about mid-September, when the first signs of hardening were found. In this phase the extracellular resistance r_e decreased and the β-parameter first decreased and then increased (Fig. 2A, D). In contrast, the intracellular resistance r_i and the membrane time constant τ_m first increased and then decreased (Fig. 2B, C). The change in the τ_m was more than three decades between August and September. After mid-September the τ_m stabilised at a level of about 1ms until the end of November. In the second phase, the r_e started to increase in mid-September, when a marked increase in the FH was observed (Fig. 2E). The intracellular resistance started to increase at the beginning of October, at which time the rate of hardening had considerably increased.

The parameters differed significantly between the provenances. The extracellular resistance was significantly higher and the β-factor significantly lower in the southern than in the northern provenances until about mid-September (Table 2, Fig. 2A, D). In that phase the membrane time constant was significantly higher in the southern than in the northern provenances (Table 2, Fig. 2C). The intracellular resistance was significantly lower in the southern than in the intermediate or northern provenances from the beginning of October to the end of November (Table 2, Fig. 2B).

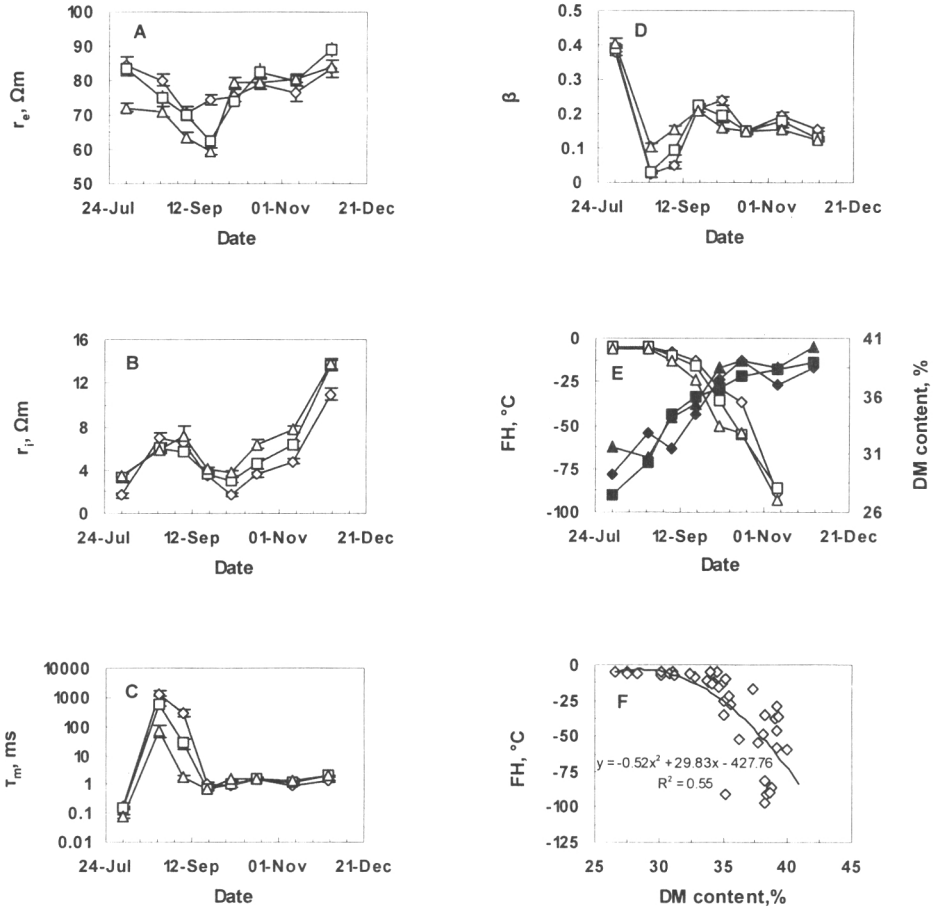

Figure 2. The impedance analysis parameters for Model-A of non-frost-exposed Scots pine needles (the samples were not artificially exposed to frost) (**A-D**), the FH of Scots pine needles assessed by the electrolyte leakage method with controlled freezing tests (empty symbols) (the FH data is the same as in Repo et al. 2000a) and the DM content of needles (solid symbols) (**E**), and relationship between the DM content of the non-frost-exposed Scots pine needles and the FH of the needles (Pooled data of six provenances) (**F**) during the study period in a provenance field trial in Suonenjoki, Finland, in 1998. Symbols: ◊ and ♦ = the mean of the two southernmost, □ and ■ = the mean of the two intermediate, Δ and ▲ = the mean of the two northernmost provenances. The parameters r_e and r_i are the extra- and intracellular resistances, respectively, τ_m is the membrane time constant, and β is a factor controlling the spectrum skewness and impedance locus centre depression. Each point in the case of the EIS-results is the mean of two provenances (n=32), 16 needles per provenance (Table 1). For FH, each point is the mean of two provenances (n=2). Bars indicate standard errors.

Table 2. Comparison of extracellular resistance (r_e), intracellular resistance (r_i), membrane time constant (τ_m), and parameter β (a factor controlling the spectrum skewness and impedance locus centre depression) in the non-frost-exposed Scots pine needles (the samples were not artificially exposed to frost) between different groups of provenances at different times with Univariate Analysis of Variance. A: southern vs. intermediate group, B: intermediate vs. northern group, and C: southern vs. northern group.
df (non-frost-exposed parameters) = 31. * 0.05, ** 0.01, *** 0.001. _ not significant.

Paired group	Parameter	Sig. (Contrast Results) at different measuring times							
		3-Aug	24-Aug	7-Sep	21-Sep	5-Oct	19-Oct	9-Nov	30-Nov
A	r_e	_	*	_	***	_	_	_	*
	r_i	**	_	_	_	**	*	**	***
	τ_m	_	***	***	_	_	_	_	_
	β	_	_	***	_	***	_	_	*
B	r_e	***	_	**	_	*	_	_	*
	r_i	_	_	**	_	_	***	**	_
	τ_m	_	***	_	_	_	_	_	_
	β	_	***	***	_	**	_	*	_
C	r_e	***	***	**	***	_	_	_	_
	r_i	***	*	_	_	***	***	***	***
	τ_m	_	***	***	_	_	_	_	_
	β	*	***	***	_	***	_	***	**

No direct correlation was found between the equivalent circuit parameters of the non-frost-exposed needles and the FH in the pooled data for the whole experimental period. However, a linear relationship was found between the cessation of needle elongation (see Repo et al. 2000b) and the time for τ_m to drop to a level of 10ms between end of August and mid-September (R^2=0.58) (Fig. 2C). Furthermore, from early-October to late-November the intracellular resistance r_i increased concomitantly with the FH of the needles (R^2=0.51)

(Fig. 2B, E). The northern group attained an intracellular resistance of 5 Ωm and FH of –52°C on 12 October, while the intermediate and southern group did the same 11 and 27 days later, respectively (on 12 October, with FH of –45°C and –32°C, respectively). When the r_i of the intermediate and southern groups reached 5 Ωm, the corresponding FH was –62°C and –89°C, respectively.

FH of needles on the basis of controlled freezing tests

In August the FH of the needles in the different provenance groups varied between –5°C and –7°C and there were no significant differences between the groups. The hardening of the needles was initiated first in the northern group, at the beginning of September, and then in the intermediate and southern groups in mid-September (Fig. 2E). The differences between the groups were significant ($P<0.05$) in September and October. At the beginning of November the FH of the needles was around –90°C. There were no significant differences between the groups in November (Fig. 2E).

DM content of needles

The DM content of the needles gradually increased in all groups during the study period (Fig. 2E). In mid-October the DM content reached its maximum value, which was roughly 37-39% of fresh weight (Fig. 2E). No significant differences in DM content between the provenance groups were found other than between the intermediate and northern groups in October.

The relation between the DM content and the FH of the needles was quadratic ($R^2=0.55$) (Fig. 2F). The FH remained fairly constant when the DM content increased substantially from 25% to 34%. The FH started to increase further when there was also an increase in the DM content up to about 39%.

DISCUSSION

Relationship between the FH and EIS parameters of needles

The results of the study support our hypotheses that the properties of Scots pine needles, as determined by electrical impedance spectroscopy, change during cold acclimation and that there are differences in the EIS properties between the provenances. No direct relation between the FH and EIS parameters of Model-A was found, however. Instead, according to the changes in the EIS-parameters, there were clearly different phases in the cold acclimation indicating the sequence of events taking place in needles in the maturation and cold acclimation phase.

The shape of the impedance spectra of the needles changed during the study period. There were two arcs in early August, when needle elongation was ceasing, and a single arc from September until the end of the study period. This differs from the results of former studies, where only one arc was found in Scots pine needles (Repo et al. 1994, Zhang et al. 1995). In the previous studies, fully developed needles were measured on only one or two occasions. It is probable that the structure of the needles changes during the maturation phase in August, which would then affect the features of the impedance spectra. The impedance arcs typically had depressed centres and the spectra were slightly skewed in accordance with the findings of previous studies of Scots pine (Repo et al. 1994, Zhang et al. 1995).

According to the CNLS analysis of the spectra using the Model-A, all of the impedance parameters of the non-frost-exposed needles changed during the study period from early-August to late-November. In August and September the parameters r_e, r_i and β varied without displaying any direct relation with frost hardiness. This contradicts the results of stems where the intracellular resistance and the relaxation time correlated with the FH (Repo et al. 1997, 2000a).

In August and mid-September the most substantial change occurred in the cell membrane time constant τ_m, which initially increased and then decreased. According to the previous study needle elongation had ceased in all provenances until 3 August (Repo et al. 2000b). After that date, changes are chemical and biochemical which are reflected as a change in ultrastructure. These changes satisfy the prerequisites for cold acclimation. It is

presumed that the needles were fully matured by the end of August, and that they were then ready to undergo freezing-stress.

The change in the τ_m reflects a change in the cell membrane properties in the process of cold acclimation. Since $\tau_m = R_3 \bullet C_m$, the changes in the τ_m are due to a change in the specific membrane resistance R_3, while the specific membrane capacitance C_m is assumed to be a constant of 1 $\mu F/cm^2$ (Zhang et al. 1995). Several changes occur in cell membranes during cold acclimation which may affect their stability and fluidity and thus contribute to the resistance of the cell membrane. The increase in freezing-stress resistance during cold acclimation in the autumn takes place in parallel with an increase in the proportion of 18:1 and 18:2 fatty acids in cell membranes (Sutinen 1992, Repo et al. 1997), which may in turn be related to the increase in the fluidity of the plasma membrane. The increase in the activity of the plasma membrane ATPase during cold acclimation coincides with changes in the phospholipid fatty acid composition of the plasma membrane (reviewed by Sutinen 1992). The plasma membrane becomes folded and numerous microvesicles appear, which suggests preparations being made by the cell to counter the mechanical stress caused by dehydration forces and a concomitant collapse of the cell wall as a result of extracellular freezing (Sutinen 1992). An accumulation of phospholipids and galactolipids during cold acclimation has also been found in both spruce and pine needles (Bervaes et al. 1972, de Yoe and Brown 1979, Öquist 1982, Senser 1982, Senser and Beck 1982). All these factors may in one way or another affect the cell membrane resistance.

The τ_m decreased with the very first appearance of frost hardening between the end of August and mid-September (Fig. 2C, E). The timing of this fall in τ_m followed a latitudinal pattern, i.e., it took place first in the northernmost provenance and last in the southernmost provenance in accordance with the increase in FH. According to our previous study, the needle elongation of these saplings ceased first in the northernmost provenance and last in the southernmost provenance (Repo et al. 2000b). The timing of the cessation of needle elongation also bore a clear relationship with the frost hardiness at –15°C (Repo et al. 2000b). Accordingly, we may conclude that during the initiation phase of frost hardening the membrane time constant τ_m is indirectly related to the FH.

The extracellular resistance r_e decreased until mid-September, while the intracellular r_i first increased and then decreased. The changes in the extracellular resistance r_e were similar to those in the one-year-old needles of Scots pine seedlings (Repo et al. 1984).

During the early pre-hardening phase, i.e., in August, the extracellular spaces in Scots pine needles may hold less water than that contained in the intracellular spaces. This may, in turn, cause a higher extracellular resistance at the start of the study. Because of the semipermeable characteristics of the plasma membrane, the cell behaves as an osmometer (Steponkus 1984). In the early stage of cold acclimation the water permeability of the plasma membranes begins to increase. Thus, the water content in the intracellular spaces decreases or the proportion of water decreases due to accumulation of some substance, while in the extracellular spaces it increases. This may initially cause the r_i and r_e to respond in opposite ways. Subsequently, in September, following a substantial increase in the DM content, which probably means a large reduction in the water content, the cold acclimation results in the stabilization of the plasma membrane, and an equilibrium between the intracellular and extracellular solutions results.

In the phase of substantial increase in FH, i.e., from early-October to late-November, the intracellular resistance r_i increased with the FH. The r_i between origins differed significantly, especially between the southern and intermediate groups, as well as between the southern and northern groups. The electrical resistance is inversely related to the concentration of electrolytes and their mobilities. As has been found previously, sugars accumulate in cells during hardening (Hodge and Weir 1993, Ögren 1999). These substances are low mobility electrolytes. An increase in the sugar concentration without a commensurate increase in the high-mobility electrolytes would result in a net increase in the cytoplasmic resistance, as was found in this study (cf. Colombo and Blumwald 1992). In addition, cellular compartmentalization increases during cold hardening, and this effect, combined with an increase in viscosity due to the accumulation of sugars and other cellular constituents (Stout 1988), may result in a reduction in ion movement and an increase in intracellular resistance. In this particular phase the DM content changed only slightly.

Relationship between FH and DM content of needles

The DM content of the needles started to increase before the onset of frost hardening, but there were no significant differences in DM content between the different provenances. After the DM content of the needles had reached a level of approximately 32%, the rate of hardening increased, as had also been found earlier (Junttila and Kaurin 1990, Toivonen et al. 1991, Sutinen 1992, Repo et al. 1997, 2000a). The decline in the water content is

necessary for metabolic adjustment to a low temperature and is also a prerequisite for hardening. A reduction in water content during the autumn is to some extent related to an increase in freezing-stress resistance (Levitt 1980, Sutinen 1992). The extent of this reduction varies according to species and tissues (Harrison et al. 1978, Kincaid and Lyons 1981, Smit-Spinks et al. 1984, Toivonen et al. 1991, Repo et al. 1997, 2000a, Sutinen et al. 2000).

In conclusion, the EIS analysis of Scots pine needles indicates that a sequence of events occurs during the maturation and cold acclimation phases. In particular, the cell membrane time constant and the intracellular resistance changed during these phases. EIS is simple and fast method. Hence, it is a useful means for recording the changes that occur in needles in the course of cold acclimation.

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Appendix 1.

List of symbols and abbreviations:

Symbol	Explanation	Unit
$\omega=2\bullet\pi\bullet f$	angular velocity	rad/s
$\tau_m=R_3\bullet C_m$	membrane time constant in Model-A	s
β	a factor in Model-A controlling spectrum skewness and impedance locus centre depression	
A	coefficient to define the asymptote of the sigmoid function	%
a	thickness of the needle	m
A_c	cross-sectional area	m^2
B	slope of the sigmoid function at the inflection point	%/°C
b	width of the needle	m
C	inflection point of the sigmoid function	°C
C_m	specific membrance capacitance	$\mu F/cm^2$
CNLS	Complex Non-linear Least Squares	
D	coefficient to define the asymptote of the sigmoid function	%
DM	dry matter content: (dry weight/fresh weight)•100	%
EIS	electrical impedance spectroscopy	
f	alternating current frequency	Hz
FH	frost hardiness	°C
j	complex number operator, $\sqrt{-1}$	
l	length of the needle	m
L_1	conductivity	µS/cm
L_2	conductivity	µS/cm
R_∞	high frequency resistance in Model-A	Ω
R^2	coefficient of determination	
R_3	specific membrane resistance	Ωcm^2
R_e	extracellular resistance	Ω
r_e	specific extracellular resistance	Ωm
REL	relative electrolyte leakage	%
R_i	intracellular resistance	Ω
r_i	specific intracellular resistance	Ωm
R_o	low frequency resistance in Model-A	Ω
R_x	non-specific resistance	Ω
r_x	specific resistance	Ωm
Z	impedance	Ω

IV

COLD ACCLIMATION IN SCOTS PINE: A TEST OF ADDITIVE RESPONSE AND STATIONARITY OF FROST HARDINESS BY PHOTOPERIOD AND TEMPERATURE

Gang Zhang[1,4], Aija Ryyppö[1], Elina Vapaavuori[2] and Tapani Repo[3]

[1] Faculty of Forestry, University of Joensuu, P.O. Box 111, FIN-80101 Joensuu, Finland Tel: +358 13 251 4442, +358 13 251 5256, Fax: +358 13 251 4444, E-mail: gang.zhang@forest.joensuu.fi, Aija.Ryyppo@forest.joensuu.fi

[2] The Finnish Forest Research Institute, Suonenjoki Research Station, Juntintie 40, FIN-77600 Suonenjoki, Finland, Tel: +358 17 513 811, Fax: +358 17 513 068, E-mail: elina.vapaavuori@metla.fi

[3] The Finnish Forest Research Institute, Joensuu Research Centre, P.O. Box 68, FIN-80101 Joensuu, Finland, Tel: +358 13 251 4136, Fax: +358 13 251 4567, E-mail: tapani.repo@metla.fi

[4] Current address: The Finnish Forest Research Institute, Suonenjoki Research Station, Juntintie 40, FIN-77600 Suonenjoki, Finland, Tel: +358 17 513 811, Fax: +358 17 513 068, E-mail: gang.zhang@metla.fi

ABSTRACT

A growth chamber experiment was carried out for second-year Scots pine (*Pinus sylvestris* L.) seedlings to test the following hypotheses: i) the frost hardiness of different organs is an additive property of the photoperiod and temperature, and ii) a discrete stationary level of frost hardiness prevails for those environmental factors. There were three treatments involved in the trial: a short photoperiod (7/17h; day/night) with a high temperature (15°C) (SDHT), a short photoperiod (7/17h; day/night) with a low temperature (2°C) (SDLT), and a

long photoperiod (16/8h; day/night) with a low temperature (2°C) (LDLT). Shoot elongation had ceased when the treatments were started. The frost hardiness of stems, needles, buds and roots was assessed by controlled freezing tests followed by measurement of electrolyte leakage (EL) (needles, buds and roots), and electrical impedance (EI) (stems). The EI parameters of the stems, the maximum photochemical efficiency of photosystem II (F_v/F_m ratio) of the needles, and the total soluble sugar concentration of the stems, needles and roots in the non-frost-exposed organs were measured. There was a clear difference in hardiness between the organs by the end of the experiment but no difference between the treatments in the stems, needles and buds. In the roots and buds (except in SDHT treatment) a stationary level of frost hardiness was reached, whereas in case of the stems and needles the frost hardiness was asymptotically approaching the respective stationary level. This proves that the discrete stationary level of frost hardiness according to photoperiod and temperature may be found during cold acclimation. Very little support was found for the concept of additive effects by photoperiod and temperature in all organs. In the initial phase, hardening of needles was driven predominantly by the photoperiod, while the hardening of stems was driven mainly by temperature. The hardening of roots was mainly driven by temperature throughout the treatments. The sugar concentration in the different organs followed the sequence needles > stems > roots, which also matched the levels of frost hardiness. The results show that the photoperiod and temperature are important factors affecting frost hardening of Scots pine. The additive model needs revision since the hardening responses by the factors are dependent on organ and there is an interaction in the hardening responses to photoperiod and temperature.

Keywords: Chlorophyll fluorescence, dry matter content, electrical impedance, electrolyte leakage, frost hardening, modelling, *Pinus sylvestris*, sugar concentration.

INTRODUCTION

The frost hardening of trees is a gradual process controlled by the shortening photoperiod and declining temperature. The influence of the photoperiod and temperature on frost hardening may be mediated by independent physiological processes (Siminovitch et al. 1967, Levitt 1972, Senser and Beck 1982), and the actual frost hardiness can be induced as an additive effect of these factors (Chen and Li 1978, Greer 1983). Evidence of this kind of additive behaviour has been found in controlled experiments with several woody species (Aronsson et al. 1976, Bervaes et al. 1978, Chen and Li 1978, Christersson 1978, Greer 1983). The environmental control of frost hardiness can be considered by assuming that for each combination of environmental factors there is a discrete level of metabolic status determining frost hardiness. Thus, a change in frost hardiness would occur after a certain

delay whenever the environment changes (Repo et al. 1990, Repo 1993, Leinonen et al. 1995).

Frost hardiness, as determined by controlled freezing tests, is an integrated variable of several physiological processes during cold acclimation. We have recently observed changes in the electrical properties of tissues that have been coincidental with frost hardening (Repo et al. 2000). During hardening, the dry matter content increases (Ögren 1999, Repo et al. 2000) and soluble sugars accumulate in woody plants as a result of starch hydrolysis (Sakai and Yoshida 1968). The soluble carbohydrate concentration correlates positively with frost hardiness (Ögren 1999, Palonen 1999). The decline in the maximum photochemical efficiency (F_v/F_m) of the photosystem II (PS II) is promoted by low and freezing temperatures (Öquist et al. 1987, Smillie et al. 1988, Krause 1994). The decrease in F_v/F_m ratio is indication of photoinhibition (Öquist et al. 1992, Mohammed et al. 1995), and plants subjected to a low temperature and high irradiance environments often display significant photoinhibition (Strand and Öquist 1988). We may assume that, if the concepts of stationarity and additivity hold, they may also appear in the physiological attributes underlying frost hardiness.

Several mathematical models for frost hardiness have been published in the literature. The development of such models is valuable for frost hardiness research since they enable researchers to increase their quantitative understanding of the factors that contribute to frost hardiness. The present frost hardiness models are both species- and organ-specific (Anisko et al. 1994). Basically, two main categories of models can be distinguished, i.e. regression models and dynamic models. The regression models have been developed for investigating the frost hardiness of trees by estimating the relationship between selected environmental variables and the measured frost hardiness. They are useful for predicting the development of frost hardiness for different tree species and provenances (Leinonen 1997).

The dynamic models can realistically describe the actual development of frost hardiness as far as the environment is concerned. In the models developed previously, air temperature and photoperiod have been used to predict the development of frost hardiness in Scots pine (*Pinus sylvestris* L.). In the case of one of these earlier models the effect of temperature and photoperiod on frost hardiness was considered additive (Leinonen 1996). The change in the hardening response has been modelled using the concept of hardening

competence (C_R), since the frost hardening of trees induced by photoperiod and temperature is dependent on the annual stage of development (Chen and Li 1978, Greer 1983, Leinonen et al. 1995, Leinonen 1996, Leinonen et al. 1996), i.e.

[1] $$\hat{R}(t) = \hat{R}_{\min} + C_R \cdot (\Delta \hat{R}_T + \Delta \hat{R}_P)$$

where $\hat{R}(t)$ is a stationary level of frost hardiness, $\hat{R}_{\min}$ is the minimum level of frost hardiness with no hardening induced by environmental factors, $\Delta \hat{R}_T$ is the increase in frost hardiness induced by temperature, and $\Delta \hat{R}_P$ is the increase in frost hardiness induced by photoperiod. The hardening competence is low (C_R=0) in the active growth phase, and it increases to 1 towards the end of the lignification phase (Leinonen 1996). According to the model [1] the frost hardiness induced by the photoperiod and temperature have their own stationary levels, which are assumed to be additive. The stationary level of the frost hardiness in relation to the photoperiod and temperature was assumed to be piece-wise linear (Leinonen 1996). Little quantitative experimental data exists on different organs or tissues which might lend support to these assumptions.

A minor amount of information that can be used as a basis for modelling is available about the differential hardiness of organs (buds, stems, needles and roots). Existing knowledge about root tissue hardening under different thermo- and photoperiods is also limited (Smit-Spinks et al. 1985). It has been found, for example, that an extended photoperiod and a warm temperature interfere with root frost hardiness in *Potentilla fruiticosa* L. cv. Katharine Dykes and white spruce (*Picea glauca* (Voss)) (Johnson and Havis 1977). Seasonally short days and near-freezing temperature were necessary for the maximum rates of hardening of roots to be achieved. However, Tremblay and Lalonde (1987) have shown that in the case of alder (*Alnus*) the ambient temperature is the principal determinant factor and that the photoperiod has no significant effect on the cold hardiness of its roots. These results are consistent with those obtained for *Pinus sylvestris* by Smit-Spinks et al. (1984), who showed that in their study the shoot acclimation did not trigger root acclimation. However, no mathematical model is available for the cold hardiness of roots, although this is urgently needed in the face of expected climate changes (Bigras et al. 2001).

In the present study strictly controlled environmental conditions were used to determine the dependence of frost hardiness on the photoperiod and temperature. Our aim was to test the following hypotheses: 1) the cold acclimation in buds, stems, needles and roots is an additive property driven by photoperiod and temperature; 2) the frost hardiness of buds, stems, needles and roots will attain a discrete stationary level corresponding to a given photoperiod and temperature. Another aim was to examine whether the frost hardiness of the different organs respond similarly to the environmental factors or not by assessing the electrical impedance, dry matter content, F_v/F_m ratio and sugar concentration.

MATERIALS AND METHODS

Plant material

Second-year seedlings of Scots pine (*Pinus sylvestris* L.) of central Finnish origin (61°42'N, 24°56'E, 160 m a.s.l.) were used in the experiment. The seeds originated from the seed orchard SV88 (central Finland). The seeds were sown in a greenhouse in plastic containers (container type PL 81F, growing density 546 seedlings/m^2, and cell volume 85 cm^3, Lännen Tehtaat, Finland) on 17 May 1999 at the Research Nursery of the Suonenjoki Research Station of the Finnish Forest Research Institute (62°39'N, 27°03'E, 140 m a.s.l.). The growing medium was commercial low-humidified sphagnum peat (M6, Kekkilä Co., Finland) containing premixed fertilisation, i.e. 0.8 kg basic fertiliser (Peruslannoite 6, Kekkilä Co., Finland) and 2 kg magnesium-rich limestone per cubic meter of peat. The 'Peruslannoite 6' contains 16% N, 8% P and 16% K plus micronutrients. The cells were thinned to one seedling per cell on 1 June 1999. In the course of 1999, liquid fertilisation was applied four times (15 June, 28 June, 8 July and 20 July) using the fertiliser Superex 9 (Kekkilä Co., Finland) 5 g m^{-2}; and once (28 July) using the fertiliser Superex 5 (Kekkilä Co., Finland), 5 g m^{-2} in irrigation water. By including the premix fertiliser in the peat medium, the seedlings were given 24 mg N, 10 mg P and 26 mg K per seedling during the first growing season in 1999. On 23 July 1999, the seedlings were moved out from the greenhouse to winter in the nursery field.

During the second growing season in 2000, the seedlings continued to grow in the same containers as they had in their first season in the research nursery. The seedlings were

raised using normal nursery routines from 2 May to 2 July. They were fertilised three times, i.e. 7 June, 20 June and 29 June, using a 0.1% solution of Kekkilä Superex 9 corresponding to 11 mg N, 3 mg P and 11 mg K per seedling. On 3 July, 4374 seedlings (54 trays × 81 seedlings/tray) were transferred to the University of Joensuu (62°36'N, 29°43'E, 81 m a.s.l.) for the start of the experiment in the growth chambers.

Treatments

In Joensuu, the seedlings were divided between six growth chambers (four PGW36 and two DePGW36; Conviron, Canada) for the three treatments, with two replicate chambers for each. From 3 July to 10 July the seedlings were kept under similar conditions, i.e. 20/15°C temperature (day/night), 18/6h photoperiod (day/night), 200 μmol m^{-2} s^{-1} photon flux density and 60/80% relative humidity (day/night). The treatments with different photoperiods and temperatures started on 11 July 2000. Three treatments were used: a short photoperiod (7/17h; day/night) with a high temperature (15°C) (SDHT), a short photoperiod (7/17h; day/night) with a low temperature (2°C) (SDLT), and a long photoperiod (16/8h; day/night) with a low temperature (2°C) (LDLT). Each seedling was watered according to the weight of water lost from its state of saturation. No fertiliser was applied during these treatments.

Morphological measurements

The stem diameter 1 cm above the peat surface, the shoot elongation (growth in height), and needle elongation were measured weekly using a calliper, from 11 May to 4 August 2000. At the Suonenjoki Research Station, 5 Plantek-81 trays containing 10 seedlings per tray (a total of 50 sampling seedlings) were chosen randomly for the morphological measurements. After the seedlings were transferred to the growth chambers at the University of Joensuu, 16 seedlings in a single tray in each chamber were selected according to the mean height of the seedlings for the morphological measurements to be continued, i.e. their stem diameter, height growth and needle elongation. The needle elongation was monitored for a single pair of current-year needles from a central point of the current-shoot per seedling and the mean of the two values was calculated as the needle length of each sample seedling.

Measurements of frost hardiness

The frost hardiness of the buds, current-year stems, current-year needles and coarse roots was assessed by means of controlled freezing tests conducted in air-cooled chambers (ARC 300/-55+20, Arctest, Finland) and in an N_2-gas-cooled chamber (GCC-30, Carbolite, UK with Taylor-Whatron XL-180 liquid N_2-tank). Either 6 or 7 exposure temperatures with 5°C as the control were used in each test. Five tests were conducted at intervals of two or three weeks. The programmed initial and final temperature during each exposure was 5°C, and the samples of each exposure temperature were placed in a separate chamber and kept at the target minimum exposure temperature for 4 hours. The rate of cooling and warming was 5°C h^{-1}. The freezing injuries were assessed on the basis of the electrolyte leakage (EL) (buds, needles and roots), and electrical impedance (EI) (stems). The exposure temperature ranged from 0 to –80°C, depending on the organ, treatment and developmental stage of the plants. The test temperatures were decided on the basis of the results of previous tests in order to cover the assumed critical temperature range for freezing damage.

Electrolyte leakage tests for buds, needles and roots

Before each sampling, 16 seedlings were selected for each replicate of each treatment and each freezing temperature. From the seedlings, 16 buds (1 bud/seedling), 32 current-year needles (2 needles/seedling) and 32 root sections (2 sections/seedling by using the top two coarse roots of each seedling) were sampled. For the freezing tests of needles and roots, 10-mm section were dissected from the middle part of the organs and rinsed with distilled water. The needle and bud samples were placed in plastic bags, each organ separately, to run the freezing test. Distilled water was sprayed into the plastic bags to avoid excessive supercooling. The root samples were placed directly in test tubes (4 tubes/temperature with 8 sections/tube for each replicate of each treatment) with 12 ml of distilled water prior to the start of the freezing tests (Ryyppö et al. 1998).

After completion of the freezing tests, the 10-mm sections of the needles were placed in test tubes (altogether 4 tubes/temperature with 8 sections/tube for each replicate of each treatment). The bud samples were placed in 4 tubes per temperature with 4 buds per tube for each replicate of each treatment. For the buds and needles 9 and 6 ml of distilled water were added to the test tubes, respectively. The tubes were shaken at room temperature for

24 h before the first conductivity measurement (L_1). The samples were then heat-killed at 92°C for 20 min and shaken for another 24 h before the second measurement of conductivity (L_2). The relative electrolyte leakage (REL) was calculated as:

[2]

$$REL = \frac{L_1}{L_2} \bullet 100$$

The frost hardiness was estimated with the response of REL to the exposure temperature, i.e. as the inflection point (parameter C) of Eq. [3] (Repo and Lappi 1989).

[3]

$$y = \frac{A}{1 + e^{B \bullet (C - x)}} + D$$

where *y* and *x* refer to the REL and the exposure temperature, respectively, *A* and *D* define asymptotes of the function, and *B* is the slope at the inflection point *C*.

Electrical impedance analysis for stems

The frost hardiness of the frost-exposed stems was determined as the extracellular resistance of each specimen obtained by means of an electrical impedance analysis (Repo et al. 1994). Sixteen stems were used for each replicate of each treatment and each temperature. Immediately after the frost exposure and thawing, the 15-mm section cut from the central portion of the stem was used for the measurement. The section was placed directly in contact with the Ag/AgCl-cell (electrodes of the RC1 type, WPI Ltd., Sarasota, U.S.A.) using electrode paste, in order to measure an impedance spectrum at 42 frequencies between 80 Hz and 1 MHz (HP 4284 A) (Repo 1994). The input voltage level of the sine signal was 100 mV (rms). The measurement of each sample took 30 s.

A distributed circuit element model (see below) was used to calculate the extracellular resistance according to the impedance spectra (Repo et al. 1994). The electrical impedance spectroscopy (EIS) parameters of the equivalent circuit were estimated using an automated Complex Non-linear Square (CNLS) fitting program (T Repo) which uses LEVM v6.0 (JR Macdonald, Department of Physics and Astronomy, University of North Carolina, Chapel Hill, NC). The frost hardiness was estimated with the response of the log-transformed specific extracellular resistance to the exposure temperature, i.e. as the

inflection point of the logistic sigmoid function (Eq. [3]). In the equation, y and x refer to the log-transformed specific extracellular resistance and the exposure temperature, respectively. A and D define asymptotes of the function, and B is the slope at the inflection point C. The specific resistance was obtained by normalisation of the resistance in respect of the cross-sectional area and the length of the sample.

Measurements of the non-frost-exposed samples

Impedance analysis of stems

The term "non-frost-exposed samples" means that the samples were not artificially exposed to frost in controlled freezing tests. Immediately after sampling, the electrical impedance spectra of the 16 stems for each replicate of each treatment (one stem per sample seedling) at each sampling time were measured in the laboratory using the method described above. The data were modelled using an equivalent circuit with two distributed circuit elements (DCE) in series with a resistor (double-DCE model) (Repo et al. 1994). The DCE-element is composed of a constant phase element in parallel with a resistor (Macdonald 1987). The complex impedance (Z) of the double-DCE is shown as:

[4]

$$Z = R + \frac{R_1}{1 + (i \bullet \tau_1 \bullet \omega)^{\psi_1}} + \frac{R_2}{1 + (i \bullet \tau_2 \bullet \omega)^{\psi_2}}$$

where the angular velocity $\omega = 2\pi f$ (f = frequency). In the double-DCE model there are three resistances (R, R_1 and R_2), two relaxation times (τ_1 and τ_2) and two distribution coefficients (ψ_1 and ψ_2) of the relaxation times (Repo et al. 2000). The letter i refers to the imaginary unit. The parameters were estimated by means of the automated CNLS-program (see above).

The extracellular resistance (R_e) was obtained as

[5]

$$R_e = R + R_1 + R_2$$

and the intracellular resistance (R_i) as

[6]

$$R_i = R \bullet (1 + \frac{R}{R_1 + R_2})$$

The resistance parameters were normalised with respect to the cross-sectional area ($A = \frac{\pi \bullet d^2}{4}$, d = diameter) and the length (l) of the sample in order to obtain the specific resistances (Eq. [7])

[7]

$$r_x = \frac{A}{l} \bullet R_x$$

Lower-case letters have been used to indicate the normalised values.

F_v/F_m of needles

For each replicate of each treatment and each sampling time, 5 needles per seedling were sampled (altogether 80 needles). The needles were divided between 8 replicates and were then placed in a plastic bag. The ground fluorescence F_o and maximum fluorescence F_m of dark-adapted (20 min) needles were measured using a portable chlorophyll fluorometer (MINI-PAM, Heinz Walz, D-91090 Effeltrich, Germany) at 24°C immediately after sampling. The maximum photochemical efficiency of PS II was measured as the ratio of variable to maximum fluorescence (F_v/F_m), where $F_v = F_m - F_o$.

Determination of dry matter (DM) content

The fresh and dry weights of the needles and roots were measured for the pooled samples (16 samples per replicate of each treatment). The stem sections (16 samples per replicate of each treatment) were measured separately. The samples were dried at 60°C for 48 h. The DM content was calculated as the percentage of fresh weight.

Carbohydrate analyses

Soluble sugars were analysed in the needles, stems and roots as described by Hansen and Møller (1975). Briefly, after being dried at 60°C for 48 h, each of the samples was ground into a powder. The soluble sugars were extracted from the ground samples (30-40 mg dry weight) using a total volume of 15 ml of 80% aqueous ethanol. First, 3 ml of ethanol was added to the sample, 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. The total soluble sugars in the pooled supernatant were determined colorimetrically at 630 nm with

anthrone and using D-glucose (alpha-D-glucose, anhydrous, analytical grade, Serva) as a standard.

Statistical analysis

Stationary level of frost hardiness was estimated by non-linear regression of following sigmoid function (Eq. [8]):

$$[8] \qquad R(t) = \frac{\hat{R}}{1 + (\frac{\hat{R} - R_0}{R_0}) \bullet e^{-t/\tau}}$$

where $R(t)$ is frost hardiness, $\hat{R}$ is stationary frost hardiness, R_0 is initial frost hardiness at t=0, t is time, and τ is time constant. One-way analysis of variance (ANOVA) was used to calculate the data on electrical impedance parameters, DW content, F_v/F_m, sugar concentrations and frost hardiness at different times of measurements. The mean value of the replicate in each treatment was applied in all of the evaluations of the measurement data. The difference between frost hardiness for comparing the different treatments at different measuring times was taken as significant if the Wald 95% confidence intervals did not overlap (SPSS 8.0 for Windows, SPSS Inc.). In order to evaluate the reliability of the regression model, the coefficient of determination (R^2) was also examined (SPSS 8.0 for Windows, SPSS Inc.).

RESULTS

Seedling growth characteristics

Shoot elongation and diameter growth began at the Suonenjoki Research Nursery in mid-May, which was one month earlier than for the needle elongation, which started in mid-June (Fig.1). The shoot elongation of the seedlings had ceased before the treatments in the growth chambers commenced. One week after commencing the growth chamber treatments, the needle elongation in the LDLT and SDLT treatments ceased while the needle elongation in the SDHT treatment continued. A slight increase in stem diameter was still found at the end of the experiment (Fig.1).

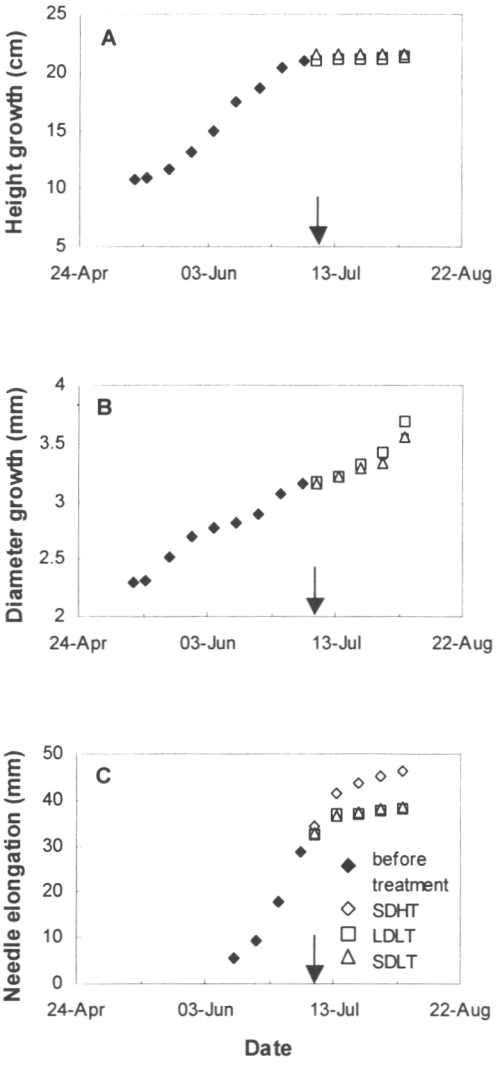

Figure 1. Second-year growth of Scots pine seedlings before and during the photoperiod and temperature treatments. Each point is the mean of 50 seedlings from 11 May to 2 July and of 32 seedlings in each treatment from 3 July to 4 August 2000. The arrow indicates the beginning of the treatments.

Frost hardiness of different organs by controlled freezing tests

The frost hardiness of stems was about –6°C before the growth chamber treatments started. The frost hardiness had an immediate response to the change in the environmental conditions, except in SDHT treatment. During the first 36 days after the start of the treatments the frost hardiness of the stems in the LDLT and SDLT treatments was about 2.5°C higher than in the SDHT treatment (Fig. 2A). By the end of the experiment the frost hardiness was about –14°C in all treatments (Fig. 2A).

Initially, the frost hardiness of the needles was –8°C. When the treatments commenced, the hardening of needles started after some delay; first in the SDHT and SDLT treatments and last in the LDLT treatment (Fig. 2B). In the stages of hardening from day 36 to day 50 the needles in the LDLT treatment were significantly ($P<0.05$) less frost-hardy than in the SDHT and SDLT treatments. There were, however, no differences in the frost hardiness of needles as to the various treatments by the end of the experiment (Fig. 2B).

Initially, the frost hardiness of the buds was also about –8°C. When the treatments started, the frost hardiness increased at a higher rate in the SDLT treatment than in the SDHT and LDLT treatments (Fig. 2C). Accordingly, in the initial stages of the experiment the buds in the SDLT treatment were significantly ($P<0.05$) more frost-hardy than in the SDHT and LDLT treatments. By the end of the experiment, no difference in frost hardiness was found between the LDLT and SDLT treatments. In contrast, the frost hardiness in the SDHT treatment was not measured correctly due to the failure of curve fitting in Eq [3].

To start with, the frost hardiness of the coarse roots was approximately –6°C (Fig. 2D). Following the commencement of the treatments, the roots in the LDLT and SDLT treatments became significantly ($P<0.05$) hardier than in the SDHT treatment, and that difference lasted until the end of the experiment. In the LDLT and SDLT treatments the frost hardiness reached a stationary level of –9.5°C in about 36 days, whereas in the SDHT treatment the hardening proceeded more slowly than in the other two treatments and remained at a lower level of –8.5°C at the end of the experiment.

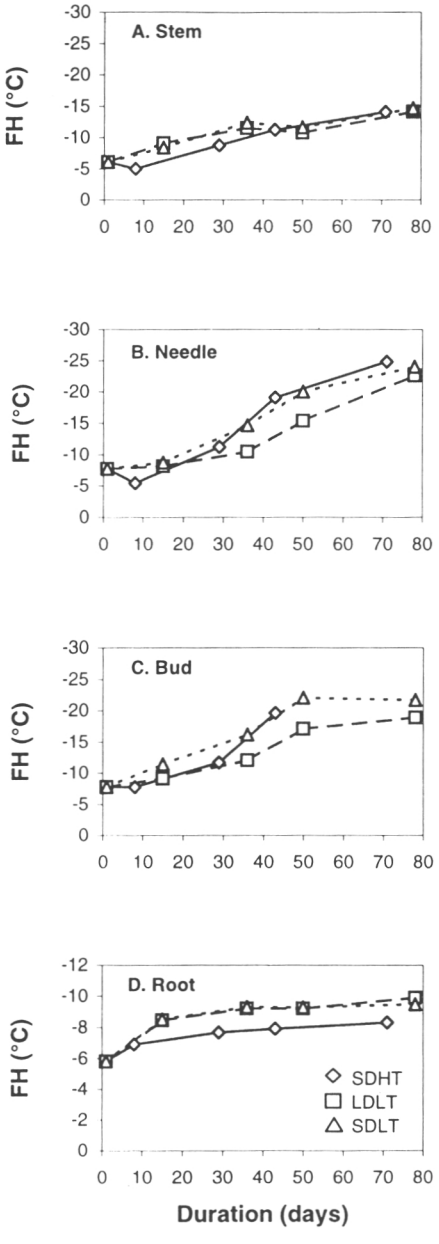

Figure 2. Frost hardiness (FH) of different organs of second-year Scots pine seedlings during the treatments. A: FH of stems assessed by electrical impedance (EI) method. B, C and D: FH of needles, buds and roots, respectively, assessed by the electrolyte leakage (EL) method. Each point is the mean of the two replicates. Day 1, beginning of treatment.

Stationary frost hardiness estimation

According to the estimation by means of the non-linear regression of sigmoid function (Eq. [8]), the stationary level of frost hardiness of each organ, i.e. stems, needles, buds and roots, was asymptotically approached in all the three treatments (except for the SDHT treatment in buds because of the failure of the frost hardiness measurement in the last measuring time). In each organ, no differences were found between treatments (Table 1). The coefficient of determination (R^2) for the fitted model ranged from 0.62 to 0.99 (Table 1).

Table 1. The asymptotical stationary level of frost hardiness (FH) in stems, needles, buds and roots estimated by means of non-linear regression of sigmoid function (Eq. [8]), and the coefficient of determination (R^2) of the fitted model in different treatments.

Treatment	Stem		Needle		Bud		Root	
	Asymptotic FH, °C	R^2	Asymptotic FH, °C	R^2	Asymptotic FH, °C	R^2	Asymptotic FH, °C	R^2
SDHT	-16.9a	0.96	-29.1a	0.96	—	—	-8.2a	0.66
LDLT	-15.7a	0.77	-33.8a[(1]	0.77[(1]	-22.4a	0.62	-9.5a	0.87
SDLT	-15.1a	0.92	-27.6a	0.97	-23.1a	0.93	-9.4a	0.99

(1: Fixed value used for the intercept in non-linear regression.
a: In each column, values carrying the same letter are not statistically different ($P<0.05$) if the Wald 95% confidence intervals overlap (SPSS 8.0 for Windows, SPSS Inc.).
— Missing value.

Impedance analysis, dry matter content, F_v/F_m measurements, and sugar concentration of the non-frost-exposed samples

Impedance analysis of stems

The time course of the relaxation time (τ_1) and the intracellular resistance (r_i) of the stems was similar for all of treatments (Fig. 3A, B). In the last assessments, both τ_1 and r_i were lowest in the SDHT treatment. The resistance ratio r_2/r_1 decreased during the treatments and no differences were found between the treatments (Fig. 3C). According to the resistance parameter of the low frequency arc (r_2), the distribution coefficient (ψ_1) of the

relaxation time τ_1, and the extracellular resistance (r_e), the stems in the SDHT treatment differed significantly ($P<0.05$, $P<0.01$ and $P<0.001$ according to parameters and treatments) from those in the other two treatments throughout the study (Fig. 3D, E, F).

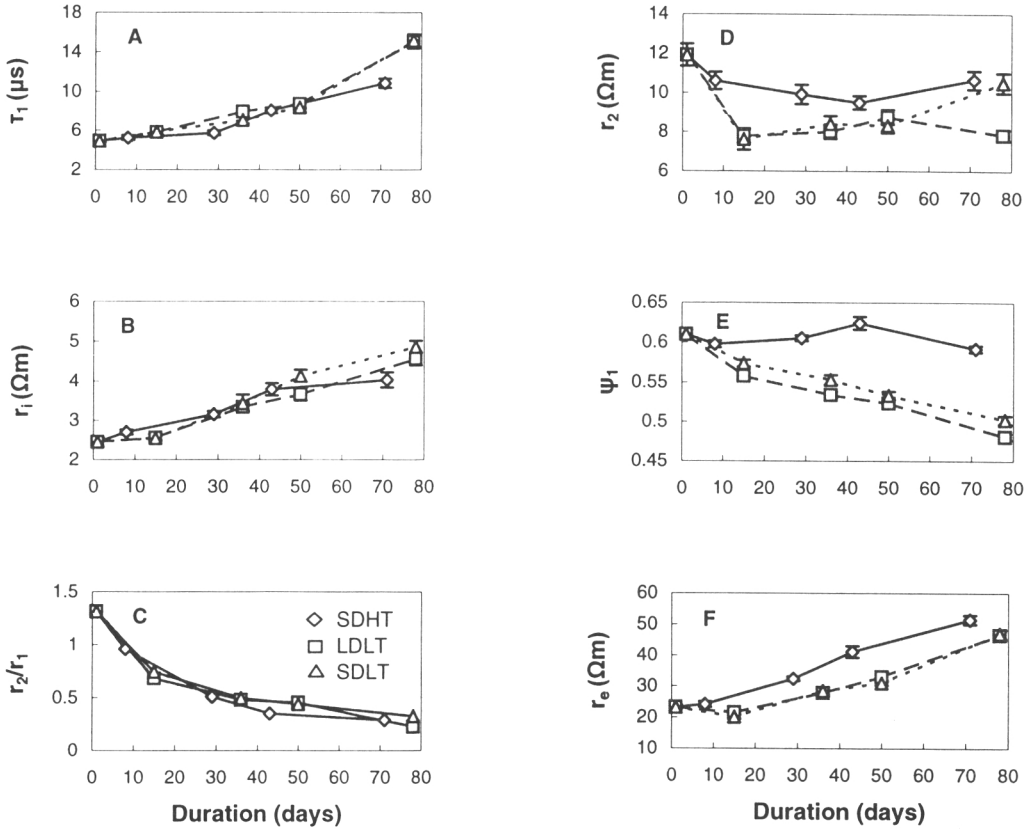

Figure 3. The electrical impedance parameters for the double-DCE model of the non-frost-exposed stems (the samples were not artificially exposed to frost) of the second-year Scots pine seedlings during the treatments. A: the relaxation time τ_1, B: the specific intracellular resistance r_i, C: the specific resistances r_2 and r_1 ratio, D: the specific resistance r_2, E: the distribution coefficient Ψ_1 of the relaxation time τ_1, F: the specific extracellular resistance r_e. Each point is the mean of two replicates (16 stems of both replicates). The bars indicate standard errors.

Dry matter content of stems, needles and roots

The DM content of the stems, needles and roots increased in all three treatments (Fig.4). In the case of the stems, there were differences ($P<0.001$) between the treatments in their initial stages, but the differences later disappeared (Fig. 4A). The DM content of the needles was lowest in the SDHT treatment (Fig. 4B). No clear difference in the DM content of the roots was found between the various treatments (Fig. 4C).

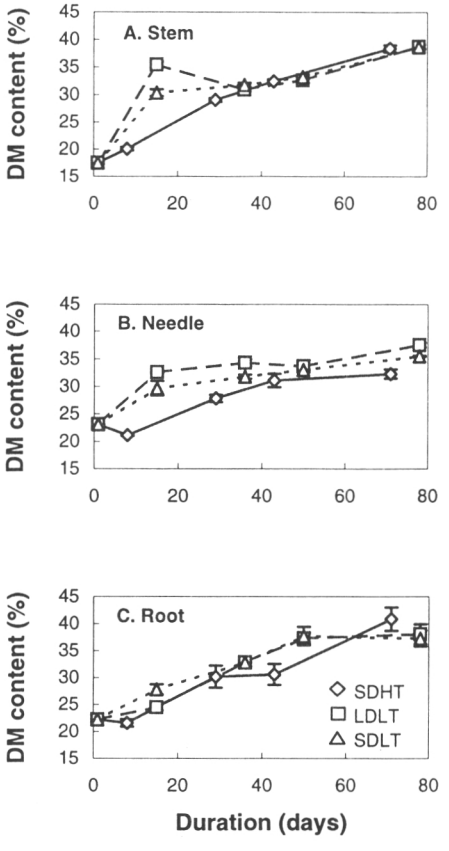

Figure 4. Dry matter (DM) content of stems (A), needles (B) and roots (C) in second-year Scots pine seedlings during the treatments. Each point is the mean of two replicates. In the case of the stems, 16 stems per replicate were measured separately (n=32), whereas in case of the needles and roots the 16 samples were pooled by replicate (n=2). The bars indicate standard errors.

F_v/F_m ratio of needles

The F_v/F_m ratio differed significantly ($P<0.005$) between the treatments. The F_v/F_m values clearly decreased in the LDLT and SDLT treatments in the course of the present study (Fig. 5). The F_v/F_m ratio was highest in the SDHT treatment, with almost no decrease during the treatment period.

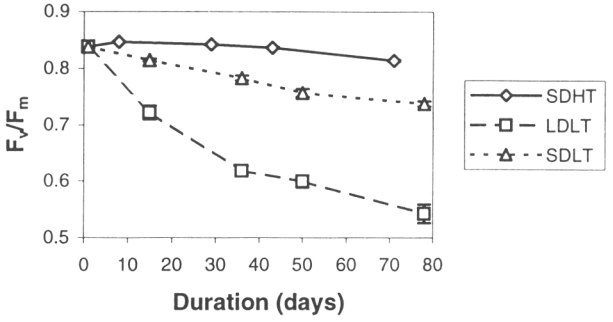

Figure 5. F_v/F_m ratio of the non-frost-exposed needles (the samples were not artificially exposed to frost) in second-year Scots pine seedlings during the treatments. Each point is the mean of the two replicates (8 F_v/F_m measurements in each replicate; 10 needles in each measurement). The bars indicate standard errors.

Sugar concentration of stems, needles and roots

The total soluble sugar concentration of the stems, needles and roots started to increase immediately after a low temperature (+2°C) was imposed (LDLT and SDLT) (Fig. 6A, B, C). In contrast, in the SDHT treatment the sugar concentration first decreased slightly and then either stayed constant or slightly increased, depending on the organ. There was no difference between the sugar concentrations in the SDLT and LDLT treatments by the end of the study. The sugar concentration remained at lowest in the SDHT treatment in the case of all of the organs.

When the sugar concentration in the different treatments was compared for the different organs, the highest level in all of the treatments was in the needles and the lowest in the roots. In most cases, the differences between the organs were significant, especially between the roots and the other two organs (in all three treatments, $P<0.001$; except in SDHT treatment, between roots and stems $P<0.01$).

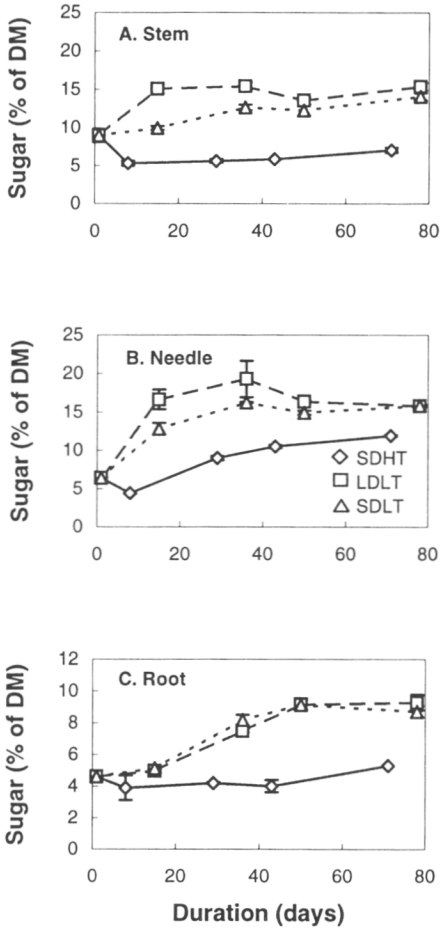

Figure 6. The total soluble sugar concentration (as percentages of dry matter content) of the stems (A), needles (B) and roots (C) in second-year Scots pine seedlings during the treatments, respectively. For stems n=6 and for needles and roots n=2. The bars indicate standard errors.

DISCUSSION

Additivity and stationarity of frost hardiness

The results of this study support our hypothesis that the frost hardiness of Scots pine reaches a stationary level when the environmental factors remain constant. This principle

held most clearly for roots and buds (except in SDHT treatment because the hardiness of the buds was not assessed accurately on the final occasion). In the case of stems and needles, the estimation of the stationary frost hardiness by Eq. [8] proved that by the end of the 78 days of the study period an asymptotical level of frost hardiness was reached.

Another hypothesis concerning the additive effect of photoperiod and temperature on frost hardiness was little supported, however. According to our hypothesis, the degree of hardiness should have been highest in the SDLT treatment, where it was assumed that the low temperature and short photoperiod would have their additive effects independently. In the stationary stage or the asymptotically approaching stationary stage none of the organs followed that principle. Only in buds on the third measuring time, the net increase in frost hardiness in the SDLT treatment was 8.4°C, which equalled the additive net increase of hardiness in the SDHT and LDLT treatments (3.9°C + 4.3°C = 8.2°C). In previous studies the additive effect of temperature and day length on frost hardiness has been found in several woody species (Bervaes et al. 1978, Chen and Li 1978, Christersson 1978, Greer 1983), and accordingly it was introduced in the frost hardiness models (Leinonen et al. 1995, Leinonen 1996, Leinonen et al. 1996). Previous results were obtained for one specific organ or for the whole seedling, without emphasising the differential responses of the separate organs or tissues. In the stems of red osier dogwood (*Cornus stolonifera* Michx.), temperature, water supply, and day length all had a significant effect on frost survival, but there were no significant interactions among them. An increase in frost hardiness resulted if any of these factors changed, and the net effect was the sum of the individual effects (Chen and Li 1978).

In roots, the short day treatment induced a 2°C increase in hardiness, from –6°C to –8°C. The low temperature alone induced hardening from –6°C to –10°C. According to the additivity principle, the hardiness should have increased by 6°C in the SDLT treatment. This was not, in fact, the case, since the hardiness in the SDLT treatment proved to be the same as in the LDLT treatment.

In the stationary state, the frost hardiness of buds was highest (–22°C) in the SDLT treatment and about 3°C less in the LDLT treatment, i.e. the low temperature alone did not induce such high hardiness as the short photoperiod and low temperature together. The additive effect could not be tested in the stationary state because the hardiness of the buds was not assessed accurately in the SDHT treatment on the final occasion. The hardening

proceeded fastest, however, when the effects of both the short photoperiod and the low temperature were simultaneous (i.e. the SDLT treatment).

The frost hardiness of the needles seemed to approach approximately the same level of –25°C in all treatments towards the end of the study, i.e. there were no additive effects produced either by short days or by low temperature for the needles in an asymptotically approaching stationary state. However, the frost hardiness of the needles responded more slowly under the influence of low temperature alone than if a short photoperiod was included, i.e. the photoperiod clearly has some impact on cold acclimation.

In the case of stems, the only difference ($P<0.05$) between the treatments was found in the initial stages of hardening. Hardening under the conditions of short photoperiod and high temperature was delayed in comparison with the other two treatments when a low temperature was included. Thus, the impact of a short photoperiod on the frost hardiness of the stems differed from that of needles, where the short photoperiod enhanced the hardening.

Low temperature played a predominant role in the hardening of stems and roots, whereas a short photoperiod played a predominant role in the hardening of needles. This is probably due to the initiation of different processes by each of those two factors in different organs. According to previous studies, temperature is the key factor for controlling the rate of hardening in Monterey pine seedlings (*Pinus radiata* D. Don) (Greer and Warrington 1982, Greer 1983, Greer et al. 2000). This conclusion is partly supported by our own results in roots and stems. However, it has been suggested that the role of the photoperiod were as important as temperature in the initial phase of hardening (Weiser 1970). Some of the processes may be hormone-dependent and may, therefore, be induced by short days. Others may trigger the activity of different enzyme systems and thus were related to temperature. If it were the sum of all these processes that determined the level of development of frost hardiness, low temperature alone should result in a certain level. Likewise, short days alone should produce their own effect, and if the two treatments are combined there should be an additive effect (Christersson 1978).

Change in the stationary level of frost hardiness in relation to different environmental factors may also occur at the beginning of the hardening phase (Leinonen 1996). Although it has been demonstrated that both low temperature and short photoperiod are separately able to induce a certain level of frost hardiness (e.g. Chen and Li 1978, Christersson 1978),

it has also been suggested that for a maximum level of hardiness plants must pass through several stages of hardening, where exposure to a short photoperiod must precede exposure to low temperature (Weiser 1970).

Impedance parameters, dry matter content, F_v/F_m ratio, and sugar concentrations of the non-frost-exposed organs related to frost hardening

The CNLS analysis of the spectra modelled using the double-DCE showed that the impedance parameters of the non-frost-exposed stems altered during the hardening treatments. The individual parameters in the different treatments changed in a similar way to that found in a provenance study of Scots pine saplings (Repo et al. 2000). No clear difference was found in the relaxation time τ_1 and the intracellular resistance r_i of stems between treatments, even though the SDHT treatment differed slightly in terms of frost hardiness from that in the other two treatments. There was no indication of additive effect by parameters τ_1, r_i, and also r_2/r_1 ratio. The other EIS parameters, i.e. r_e, r_2 and Ψ_1, showed that some treatment effects in stems were evident. These parameters were clearly different in the SDHT treatment from those in the other two treatments ($P<0.05$, $P<0.01$ and $P<0.001$ by parameters, respectively). No significant difference was found between the LDLT and SDLT treatments, proving that in second-year Scots pine seedlings the stem frost hardiness was not an additivity according to the photoperiod and temperature, but it was mainly influenced by temperatures. However, no relation was found between frost hardiness and parameters r_2 and Ψ_1, indicating that these parameters indirectly connected with frost hardiness.

The DM content of different organs increased during cold acclimation in accordance with Ögren's findings for willow stems (1999). The DM content of the needles was lower in the SDHT than in the LDLT and SDLT treatments, which was the opposite of the development of frost hardiness. This indicates that the DM content is not directly related to frost hardiness (Repo et al. 2000).

The F_v/F_m ratio of the needles in the SDHT treatment remained quite stable (0.75-0.85) throughout the study, and thus corresponded to the level of the unstressed plants (Björkman and Demmig 1987). The F_v/F_m in the LDLT treatment decreased most, indicating that the drop in the F_v/F_m was caused mainly by low, non-freezing temperatures (Öquist et al. 1987, Smillie et al. 1988, Krause 1994). In the SDLT treatment the F_v/F_m fell between the

values of the SDHT and LDLT treatments, and accordingly no additive effect was found. Further studies are needed to explain how the short photoperiod exerted its influence at a lower temperature and thus produced a higher F_v/F_m than in the low temperature treatment without using short photoperiod. In general, no correlation was found between the F_v/F_m ratio of the non-frost-exposed needles and the frost hardiness of the needles with the pooled data in this study (data not shown).

In the case of all of the organs, the sugar concentration was mainly affected by temperature. The concentration was much higher in the LDLT and SDLT treatments than the SDHT treatment. The change in the stems and roots occurred in accordance with their frost hardening (cf. Aronsson et al. 1976, Amundson et al. 1992, Ögren 1999, Oleksyn et al. 2000). When the sugar concentration in the SDHT treatment was compared for the different organs, it was found to increase only in the needles. This suggests that the needles were the target of the photoperiodic stimulus of cold acclimation in trees. In a previous study, the commencement of frost hardening could be found according to the stepwise increase in the sucrose-to-glucose ratio, and the progress of the frost hardening could be determined from the gradual rise in the total sugar concentration (Ögren 1999). Low temperature strongly intensified the change in the carbohydrate metabolism and the development of frost hardiness (Aronsson et al. 1976). In both Scots pine and Norway spruce (*Picea abies* Karst.) soluble sugar concentrations (especially sucrose) were highly correlated with frost hardiness, particularly at low temperatures (Aronsson et al. 1976). Bigras et al. (1989) observed that in Chinese juniper (*Juniperus chinensis* L.) sugars accumulated in concert with frost hardening at 1°C. According to that study, hardening was associated with little change in the sugar concentrations at 8°C and 15°C.

The sugar concentration in the stems, needles and roots reached a stationary level during the treatments. This appears to support our hypothesis that a stationary level of frost hardiness will be attained if the environmental conditions hold constant. For the stems and needles, the time required to reach the stationary level was ahead of the change in frost hardiness, while for the roots it occurred simultaneously with the frost hardening. Accordingly, it may be speculated that the stationary level or the asymptotically approaching stationary level of frost hardiness is perhaps connected with the sugar concentration.

In all three treatments the sugar concentration was highest in needles, intermediate in stems, and lowest in roots. These results accord with the frost hardiness of the same organs. In a study made by Schaberg et al. (2000) in red spruce (*Picea rubens* Sarg.) seedlings, needles generally contained the highest concentration of carbohydrates in comparison with stems and roots, and they exhibited the greatest seasonal change in carbohydrate concentration. During cold acclimation, reductions in the strength of distal carbohydrate sinks limit phloem transport away from foliage, resulting in a build-up of foliar carbon reserves. Cold-induced reductions in phloem transport itself may also contribute to the accumulation of foliar sugars (Wardlaw and Bagnall 1981, Grusak and Minchin 1989, Schaberg et al. 2000). A higher concentration of sugars in needles results in a higher level of frost hardiness in the needles compared with other organs.

In conclusion the effects of both photoperiod and temperature are important for the development of the frost hardiness of Scots pine, and including these factors in the model increases its reality. The additive models need revision, since the hardening responses to photoperiod and temperature are dependent on organ, and furthermore there is an interaction in the hardening responses to those factors. To further develop the model, experiments are needed to estimate the model parameters for different pine origins and to examine the application potential of the model for other tree species.

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