Genetic and physiological aspects of cold hardiness in *Rhododendron*

ANU VÄINÖLÄ

Department of Plant Biology
Plant Breeding
University of Helsinki
FINLAND

ACADEMIC DISSERTATION IN PLANT BREEDING

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public criticism in Viikki Biocenter, Viikinkaari 5, Auditorium 1041, on 26 May, 2000, at 12 o'clock noon.

HELSINKI 2000

Väinölä, A. 2000. Genetic and physiological aspects of cold hardiness in *Rhododendron*. 47 p. + appendices.

Keywords: acclimation, cold hardiness, frost tolerance, hardening, LT_{50}, polyploidy, *Rhododendron*

Supervisors: Professor Olavi Junttila
Institute of Biology
University of Tromsø, Norway

Professor Emeritus Peter M.A. Tigerstedt
Department of Plant Biology
University of Helsinki, Finland

Reviewers: Dr. Marja-Liisa Sutinen
Rovaniemi Research Station
Finnish Forest Research Institute

Dr. Elina Vapaavuori
Suonenjoki Research Station
Finnish Forest Research Institute

Opponent: Dr. Orville M. Lindstrom
Department of Horticulture
University of Georgia, Griffin, GA, USA

ISBN (nid.) 952-91-2120-2
ISBN (pdf) 952-91-2121-0

Electronic publication at http://ethesis.helsinki.fi/

Gummerus Kirjapaino Oy
Saarijärvi 2000

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ABSTRACT

Rhododendron is among the most popular amenity plants, but the limited cold hardiness of these plants restricts their growth in northern areas. In this study, cold hardiness of *Rhododendron* was assessed in three experiments. Deciduous azaleas and both elepidote and lepidote rhododendrons were used. In addition, the effects of growing season temperature and photoperiod on growth were studied in phytotrons. Cold hardiness was determined in controlled freeze tests using visual assessment, electrolyte leakage (EL) tests and impedance spectroscopy (IS) as methods for evaluating injury in stems and leaves. Flower bud hardiness was studied visually or with differential thermal analysis (DTA). The visual assessment data were analyzed with logit models and the quantitative data with non-linear sigmoid functions. The visually scored 50% damage correlated better with the EL tests than 10 or 90% damage. Visual ratings and specific conductance measurements of stems provided similar estimates of hardiness in most cases. The most accurate hardiness estimates were obtained by visual assessment, where all data could be combined in logit analysis rather than in separate sigmoid curve fittings for each cultivar and freezing test combination.

Hardiness of field-grown mature deciduous azaleas was followed throughout the winter 1992-93 in Minnesota. Stems were usually more hardy than florets. The difference between these organs ranged from 3 to 15°C in November to 2 - 4°C in January. Hardiness was at the maximum level in January, when some plants withstood −40°C. Stems acclimated more rapidly in the fall, but florets deacclimated earlier in the spring. Rates of deacclimation in stems and flower buds were similar between March and April, but stems were still significantly more hardy than florets in April.

The adaptability of four *Rhododendron* cultivars, two evergreen rhododendrons and two deciduous azaleas, to contrasting light and temperature conditions was studied in controlled environments representing the cool Scandinavian and warmer, more southern summers. Short day (SD, 14 h) and long day (LD, 20 h) photoperiods were combined with temperatures of 15 and 24°C. LD and 24°C enhanced the growth of subsequent flushes, the height of the flushes and the number of leaves. In the evergreen cultivars the number of leaves per shoot was predetermined by conditions during bud development. After the growing season evergreen cultivars ‘Pohjola´s Daughter’ and ‘Helsinki University’ were submitted to a hardening regime. Photoperiod and temperature during the growing season affected not only the growth but also the cold hardiness. ‘Pohjola´s Daughter’ tended to continue growth in LD or at very high temperatures, and best hardiness was attained when plants had grown in SD or at high temperature. ‘Helsinki University’ responded to SD by ceasing growth regardless of temperature. It attained better hardiness after a cool growing season, and was less sensitive to photoperiod. The hardiness of the azaleas was not determined, but according to growth rhythm *R. canadense* seems to do best in a cool climate, but azalea #89132 should acclimate in the range of climates included in this study.

The cold hardiness of diploid and corresponding tetraploid rhododendrons was compared. The leaves of the diploid clones attained better cold hardiness than those of the tetraploids. Hardiness estimates from different test methods correlated with each other, but since the sensitivity of the methods varies, IS indicated weaker hardiness than EL or visual tests. The difference between the results from IS and visual evaluation was smaller and more coherent within elepidote than lepidote clones. Furthermore, changes in extracellular and intracellular resistance in IS of unfrozen leaves during the acclimation could be used to detect changes in hardiness in elepidote, but not in lepidote clones. The stem hardiness was similar in diploids and tetraploids, but the flower buds of tetraploids were less hardy than those of the diploids. The diploids had smaller florets and smaller cells in their leaves than the tetraploids.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Väinölä, A., McNamara, S. and Pellett, H. 1997. Stem and flower bud hardiness of deciduous azaleas. Journal of Environmental Horticulture 15: 45-50.

II Väinölä, A. and Junttila, O. 1998. Growth of *Rhododendron* cultivars as affected by temperature and light. Journal of Horticultural Science and Biotechnology 73: 812-821.

III Väinölä, A., Junttila, O. and Rita, H. 1999. Cold hardiness of rhododendron cultivars grown in different photoperiods and temperatures. Physiologia Plantarum 107: 46-53.

IV Väinölä, A. and Repo, T. 1999. Cold hardiness of diploid and corresponding autotetraploid rhododendrons. Journal of Horticultural Science and Biotechnology 74: 541-546.

V Väinölä, A. and Repo, T. Impedance spectroscopy in frost hardiness evaluation of rhododendron leaves. Submitted manuscript.

LIST OF ABBREVIATIONS

Ψ	coefficient of relaxation time
τ	relaxation time
2n	chromosome number in somatic cells
ANOVA	analysis of variance
CW2	'Cunningham's White' (diploid)
CW4	autotetraploid of 'Cunningham's White'
DTA	differential thermal analysis
EL	electrolyte leakage (test)
F_1	first generation after a cross
F_2	second generation after a cross, progeny from intermating F_1
IS	impedance spectroscopy
kDa	kilo Dalton
LD	long day (20 h)
LST	lowest surviving temperature
LT_{50}	temperature at 50% lethality, other values respectively
LTE	low temperature exotherm
NMR	nuclear magnetic resonance
PSII	photosystem II
r_e	extracellular resistance
REL	relative electrolyte leakage
r_i	intracellular resistance
SD	short day (14 h)
SE	standard error
VD	visual damage
VD_{10}	visual damage to at least 10% of the leaf surface, other values respectively
x	basic chromosome number of a genus

1. INTRODUCTION

1.1. *Rhododendron*

1.1.1. Taxonomy

The genus *Rhododendron* L. of the *Ericaceae* family comprises almost 1000 species (Chamberlain et al. 1996). They range in size from tiny, mat-like plants to trees up to 30 m tall. The greatest natural gene center, with more than 300 species, is in Asia in an area ranging from Nepal along the line of the Himalayas into northern Myanmar and the provinces of Yunnan and Szechwan in South-West China (Leach 1961). Almost the same number of species is found in South-East Asia from Thailand and Vietnam to Malaysia, Indonesia and New Guinea. Japan has more than 50, North America almost 30 and Europe seven native species (Chamberlain et al. 1996). Excluding the epiphytic tropical plants of the section *Vireya*, *Rhododendron* species grow mostly in the mountainous regions of the Northern Hemisphere, and thus many of them are suitable to be grown also at much more northern latitudes.

The genus is divided into eight subgenera, and further to sections and subsections (Chamberlain et al. 1996). The large, broad-leaved evergreen shrubs or trees with large flowers comprise subgenus *Hymenanthes*, also termed the elepidotes. Lepidotes, subgenus *Rhododendron*, are usually lower bushes with smaller flowers and leaves, which are often aromatic and scaly hairs cover their above-ground parts. The plants belonging to the subgenera *Pentanthera* (deciduous), *Tsutsutsi* and *Azaleastrum* (evergreen) are called azaleas. In addition, there are four species that form three additional subgenera.

The foliage of *Rhododendron* reflects the climate and altitude of their origin, the smallest scaly leaves being found at higher elevations. These plants are often late blooming polyploids, whose flowering time enables them to survive late frosts. Eight different flower forms are known. The flower colors range from white through pink to brilliant red, from yellow to orange, from pale lavender through harsh magenta shades to almost blue. The colors consist of three pigments, anthocyanins and anthoxanthins in the vacuole and carotenoids in the cytoplasm. Flowers often have a blotch of a different color. The diameter of flowers ranges from ca. 1 cm to more than 15 cm (Leach 1961).

The basic chromosome number of the genus *Rhododendron* is 13, and most of the species and hybrids are diploids, 2n=26. Tetraploids and hexaploids, but also octoploids and dodecaploids (2n=12x=156), are found in different species (Janaki Ammal et al. 1950). In nature there is continuous gene flow between species, as they merge into each other and have subspecies and varieties. Geographical distribution, habitat and flowering time can maintain species separate (Li 1957). Since species within subgenera cross readily with each other, introgression and polyploidization are involved in the evolution of new species (Leach 1961). In the Sino-Himalaya region in particular the species are still in a plastic stage of evolution. If an ecological niche is unoccupied, a hybrid would probably arise and occupy it (Leach 1961, cf. Stebbins 1971).

1.1.2. *Rhododendron* breeding

Rhododendron is among the most popular landscape plants in Europe and North America. The cultivated types include medium size shrub species, but more often the garden forms are

hybrids from complicated cross-combinations. Normal meiosis occurs in interspecific hybrids even with complex pedigrees, and many of them are fertile. Hybrid sterility is caused by abortion of male gametes or unviable pollen often caused by mutation (Li 1957, Krebs 1997). The breeding work has resulted in plants better adapted to the "average" growing conditions of modern gardens than the species, which require similar conditions to those they have in nature.

Rhododendron species were first recommended for garden cultivation in 1629, but the first known planting in England was in 1656 (*R. hirsutum* L.), followed by three American azaleas *R. canescens* (Michx.) Sweet, *R. nudiflorum* (L.) Torr. [=*R. periclymenoides* (Michx.) Shinners*] and *R. viscosum* (L.) Torr. in 1734, *R. maximum* L. in 1736, *R. ferrugineum* L. in 1752 and *R. ponticum* L. in 1763. Asian species were introduced later: *R. dauricum* L. in 1780, *R. luteum* Sweet in 1793 and *R. chrysanthum* Pall. [*R. aureum* Georgi] in 1796. In 1753 Linné knew nine species, including then a separate genus *Azalea*. By year 1800 twelve species were in cultivation. Since Wilson's first expedition to China in 1899 some six hundred species have been introduced into cultivation (Leach 1961).

The first recorded cross of *Rhododendron* was made in 1800. The result was an "azaleadendron", a hybrid between an elepidote rhododendron *R. ponticum* and a deciduous azalea *R. nudiflorum* (Bowers 1936), which should not succeed due to taxonomic distance. Purposeful hybridizing began in England in 1810 by Michael Waterer who first crossed *R. maximum* and *R. catawbiense* Michx. In 1830's the genus attained wide interest, when also amateur breeders started hybridizing and many garden hybrids became available (Leach 1961). Nowadays, most *Rhododendron* breeding is conducted on a small scale by nurserymen and amateur breeders, who mainly seek new flower characteristics. On a larger scale, some universities and other institutions have programs devoting special attention to climatic adaptation. In 1940's North America took over from Britain as the main *Rhododendron* hybridizing region of the world (Cox 1991).

Within the genus *Rhododendron* the ability to withstand very cold temperatures varies widely, species from colder regions are hardier than those from warm regions (Kaku et al. 1980, Sakai et al. 1986, Kaku 1993). The species from the Himalayas are only hardy to around –25°C (Sakai 1982). Many species in the elepidote subsection *Pontica* are very hardy; *R. brachycarpum* D. Don ex G. Don, *R. catawbiense* and *R. maximum* for example (Sakai et al. 1986). *R. catawbiense* soon after its introduction in North Carolina in 1809 became the principal source of cold hardiness in breeding (Leach 1961). *R. yakushimanum* Nakai [*R. dergonianum* ssp. *yakushimanum* (Nakai) H. Hara] has recently become the most popular parent, and its cross with *R. smirnowii* Trautv. results in cultivars suited to cold climates (Cox 1991). The lepidote *R. minus* Michx. var. *minus* Carolinianum group has been used for its extreme hardiness, but it does not cross readily with other species. The other two most-used lepidotes are *R. keiskei* Miq. 'Yaku Fairy' and *R. dauricum* (Cox 1991). Unfortunately, fragrance seldom goes together with hardiness (Cameron 1994).

In Finland *Rhododendron* breeding was started at the University of Helsinki in 1973, and has aimed at hardy cultivars of various shapes, sizes and colors. The work is based on collections established in the 1930-50's and which have proven hardy in the semi-maritime climate of Finland (Uosukainen and Tigerstedt 1988). In the extremely cold winters of 1984/85 and

* For the species that have changed name after the publication of the reference, the name in square brackets is the currently valid one (Chamberlain et al. 1996).

1986/87 only *R. brachycarpum* subsp. *tigerstedtii* Nitzelius [=*R. brachycarpum* subsp. *brachycarpum*] was completely hardy. It is said to be the world's hardiest *Rhododendron* (Cox and Cox 1997). In addition, *R. brachycarpum, R. metternichii* Siebold & Zucc. [=*R. dergonianum* ssp. *heptamerum* (Maxim.) H. Hara] and *R. smirnowii* are hardy under normal winter conditions in south-eastern Finland (Uosukainen and Tigerstedt 1988). The hardiest progenies in the breeding program were obtained when *R. brachycarpum* subsp. *tigerstedtii* as a maternal parent was crossed with *R. smirnowii, R. metternichii*, or a Seidel hybrid (Uosukainen and Tigerstedt 1988, Uosukainen 1992). The hardiness of *R. catawbiense* or its hybrids was satisfactory only when crossed with *R. brachycarpum* subsp. *tigerstedtii* (Uosukainen 1992). *R. dergonianum* ssp. *yakushimanum* gave intermediate hardiness (Uosukainen and Tigerstedt 1988). The superiority of the continental *R. brachycarpum* subsp. *tigerstedtii* as a gene source for low temperature tolerance is best utilized for wide climatic adaptability when it is crossed with a maritime plant (Uosukainen 1992).

Recently, polyploid breeding, aiming mainly at larger flowers, has become a major focus among some *Rhododendron* breeders (Pryor and Frazier 1968, Tolstead and Glencoe 1991, Eiselein 1994, Kehr 1996, Väinölä 2000). The first published report of induced polyploid *Rhododendron* was a tetraploid *R.* 'Epoch' belonging to *R. minus* var. *minus* Carolinianum Group (Kehr 1971). Kehr (1996) also listed other, previously unpublished tetraploid cultivars.

1.2. Polyploidy

Polyploidy is the multiplication of entire chromosomal complements (euploidy) or more generally, doubling of part of the chromosomes (aneuploidy). In nature, 30 to 35% of flowering plants are polyploids (Stebbins 1971). More than 90% of them are alloploids (Dewey 1980), results of combined hybridization and polyploidization, perhaps involving also mutation and subsequent recombination (Stebbins 1971, Levin 1983). The most important mechanism leading to polyploidy may be allogamy of unreduced gametes among sexually reproducing species (Lewis 1980), and the success of polyploids in nature is due to balanced hybridity, but possibly also to genome multiplication *per se* (Stebbins 1971, Tal 1980, Levin 1983). Allopolyploids express greater enzymatic diversity than either of the parents, and they can differ significantly from their diploid ancestors (Dhawan and Lavania 1996). However, due to tetrasomic inheritance chromosome doubling has a retarding effect on evolutionary change via mutation, genetic recombination and selection (Stebbins 1971).

Compared with perennial herbs, temperate woody species have higher basic chromosome numbers and lower frequencies of polyploidy. Woody polyploids are rare, possibly because of the lack of unoccupied ecological niches, where occasionally arising polyploid individuals would be superior over their diploid progenitors, as with the herbaceous polyploids, which have invaded formerly glaciated areas. Exceptions can be found, for example, in *Salix* L. and *Betula* L., and, on the other hand, *Crataegus* L., *Rubus* L. and *Rosa* L. section *Canina*, which have colonized open areas created by human destruction (Stebbins 1971).

Although allopolyploids have been highly successful in nature, almost all man-made autopolyploids are adaptively inferior to their diploid progenitors (Stebbins 1971). The most widespread effect of polyploidy is an increase in cell size. Due to it, a general *gigas* effect is seen especially in flowers and seeds (Stebbins 1971, Lewis 1980, Levin 1983). As a consequence of this, the number of cell divisions during growth and development is reduced, and

thus polyploids grow and develop slower than the diploids from which they derived. Artificially induced polyploids are characterized by increased water content, decline in stomatal density, increase in stomatal size, lower transpiration rate, less branched growth habit, disturbed meiosis and chromosomal segregation, changed regulation mechanism and physiological imbalance (Stebbins 1971, Albuzio et al. 1978, Tal 1980, Levin 1983). Changes occur in gene action, growth substances, stability of phenotypic expression and the response of polyploid plants to stress. Autopolyploidy can effect an overall increase in enzymatic activity, isozyme diversity and alteration in flavonoid profiles etc., that may lead to enhanced production and certain qualitative changes in the biosynthesis of secondary metabolites. Polyploid populations may undergo a genetic reorganization (Levin 1983, Dhawan and Lavania 1996). Putative polyploids are often recognized as having larger pollen, larger guard cells and fewer stomata. Sometimes autopolyploids have better resistance to pathogens than the diploids from which they were derived (Levin 1983, Arseniuk 1989). In some cases induced tetraploids have been found to be more cold hardy than their diploid counterparts, but the reverse is more common (Sjøseth 1971, Stebbins 1971, Levin 1983, Dhawan and Lavania 1996, Sugiyama 1998).

The transcript levels are usually proportional to the structural gene dosage, but in a few cases gene expression has been greater or less than expected (Tal 1980, Leitch and Bennett 1997). The expression of additional copies of genes in the polyploid nucleus could be suppressed by gene silencing and the level of gene expression in a polyploid can be reduced to the diploid state (Levin 1983, Dhawan and Lavania 1996, Leitch and Bennett 1997). Also, a novel form of epigenetic gene regulation may rise (Tal 1980, Leitch and Bennett 1997). The underlying molecular events are still poorly understood.

Since the discovery of the colchicine technique (Blakeslee 1937) chromosome doubling has been used as a breeding tool also in horticulture for e.g. obtaining new ornamental characteristics. In ornamental plants induced polyploidy appears as thicker leaves and stems, a deeper green color, increased width-to-length ratio of leaves, larger and more heavily textured flowers, a longer or later flowering period, and a more compact growth habit (Pryor and Frazier 1968, Stebbins 1971, Tolstead and Glencoe 1991, Gao et al. 1996, Kehr 1996). An additional reason for doubling chromosome complements in horticultural crops is to regain fertility, or prevent hybrid sterility, before or after interspecific crosses between plants of different ploidy levels, for example when the result would otherwise be a sterile triploid. Several authors have suggested producing tetraploids for using in breeding work with natural tetraploids (Kehr 1966, Pryor and Frazier 1968, Dewey 1980, Goldy and Lyrene 1984). The breeding aim can also be to produce sterile triploid plants by crossing diploids with induced tetraploids. Sterility is a desirable characteristic in clonally produced ornamental plants that normally produce messy fruits that may fall on park roads or sidewalks.

Disadvantages of artificial polyploids are more brittle wood in trees and shrubs, and slowed development and meiotic irregularities that may reduce the fertility (Stebbins 1971, Kehr 1996, Hancock 1997). In horticulture, the method has mainly been restricted to floral crops since the 1950's (Sparnaaij 1979, Hancock 1997). The use of induced polyploids in woody perennials has been limited mainly because of the long juvenile period before flowering. However, induced polyploids of *Ribes* L. and *Rubus* have been used as genetic bridges in wide interspecific crosses (see Hancock 1997).

1.3. Plant growth in the North

Thermoperiod and photoperiod of northern latitudes differ greatly from those of more southern regions, as illustrated in Figures 1 and 2 in paper II. Generally, the more northern the location, the shorter is the growing season. It starts later in spring and ceases earlier in fall. Furthermore, the temperature is never very high; for example, in Finland 35°C would be exceptional.

Photoperiod has a crucial effect on growth and dormancy development of woody plants (Wareing 1956, Heide 1974, Bigras and D'Aoust 1993). This is especially important under northern conditions at high latitudes (Junttila and Kaurin 1990), where the seasonal changes in photoperiod are dramatic varying from very short to very long days. In many species, short day conditions, as opposed to long days, result in reduction of extension and leaf growth, number of leaves, stem diameter, root and shoot dry mass production, duration of growth, earlier time of leaf abscission, later bud break and seed germination (Wareing 1956, Vaartaja 1959, Håbjørg 1972, 1978, Bigras and D'Aoust 1993). Under long days the bud formation and onset of dormancy are delayed. Furthermore, certain species – including the genus *Rhododendron* – that grow in flushes may produce several flushes under long-day conditions, though they only produce a single flush under short days (Olmsted 1951, Wareing 1956, Collin et al. 1996).

Photoperiodic ecotypes are common in trees, especially in the species with a wide north-south range in the Northern Hemisphere (Vaartaja 1959, Håbjørg 1972, Sakai and Weiser 1973, Heide 1974). The farther north the origin, the greater is the photoperiodic sensitivity and the longer the critical daylength for cessation of growth. For example, the plants at latitudes from 33 to 41°N were found to be less sensitive than the more northern ecotypes (Vaartaja 1959). Håbjørg (1978) noted that plants of different species collected within the same geographical area had approximately the same critical photoperiod for shoot elongation. Photoperiodic ecotypes of trees may be considered as evolutionary adaptations to climate (Vaartaja 1959, Håbjørg 1972). Håbjørg (1972, 1978) found the effect of latitude more significant than that of altitude. Furthermore, in maritime plants the critical daylength appeared to be shorter than in the continental plants from the same latitude (Vaartaja 1959, Sakai and Weiser 1973). Vaartaja (1959) advised taking the photoclimate into account when transferring trees, even if the thermoclimate is the same as at their origin.

Photoperiodic effects can be modified by temperature (Pettersen 1972, Barrick and Sanderson 1973, Heide 1985). Increased temperature may cause a more rapid growth cessation as a response to SD or an increasing growth rate in LD (Håbjørg 1972, Heide 1974). Temperature has a minor effect on the critical photoperiod, and can be regarded as a safety system (Heide 1974, Koski and Sievänen 1985). However, in plants where cessation of growth is not controlled primarily by photoperiod, temperature may have a significant effect on timing of growth cessation. Furthermore, the reduced number and size of leaves in *Rhododendron macrophyllum* D. Don ex G. Don at high elevation habitats may be an adaptation to cooler temperature, a shorter growing season and a deeper and longer lasting snowpack (Gholz 1978). Growth pattern and dormancy are additionally affected by endogenous rhythm (Vaartaja 1959, Levitt 1980).

Plant growth and development are also affected by shading. Shade changes both the irradiance and the spectral composition of light, for example red:far red ratio. The overall growth of *Rhododendron maximum* was found to be better the more light that was available (Nilsen

1986, Nilsen and Bao 1987). Leaves were smaller in strong light under an open sky than under deciduous or evergreen canopy, but the number of leaves, shoot length and chlorophyll content per leaf area were greater. As *R. macrophyllum* in exposed areas had less than half of the total leaf area of shaded plants, Gholz (1978) suggested that due to excess of light they have restricted the photosynthesizing-transpiring surface area. The leaf size of evergreen azaleas correlated similarly with shade treatment (Pokorny 1967). Shading increased the longevity of leaves, but more flower buds were produced in open areas (Nilsen 1986). In a natural habitat in Virginia growth was found to initiate and cease during a photoperiod of 14.4 to 15 h, earlier under an evergreen canopy than under a deciduous canopy. The growth was linearly related to photoperiod and air temperature (Nilsen 1986).

1.4. Plant cold hardiness

Cold hardiness in plants is a wide research area ranging from molecular biology through physiological studies to winter survival of plants in field experiments. In this paper cold hardiness is discussed concentrating on woody plants, especially *Rhododendron*.

The cold hardiness of plants changes seasonally (Levitt 1980). Morphology, developmental and physiological processes, as well as complex environmental interactions are all involved in these alterations (Guy 1990). Cold acclimation or hardening is the ability of an individual to survive seasonal changes in temperature without genetic changes, whereas adaptation occurs at the population level in response to changes in gene and genotype frequencies following selection pressure (see Koski and Selkäinaho 1982, Hällgren and Öquist 1990).

Dormancy is not necessary for cold acclimation, but it and hardiness can develop independently (Irving and Lanphear 1967, van Huystee et al. 1967, Aronsson 1975, Christersson 1978, Bigras et al. 1989). Kaku et al. (1983) reported independent development of hardiness and dormancy in evergreen azaleas. However, in many species growth cessation is regarded as a prerequisite for cold acclimation (van Huystee et al. 1967, Weiser 1970, Siminovitch 1981, Junttila and Kaurin 1990).

Winter hardiness refers to a plant's ability to survive all the stresses of a winter environment without injury (Graham and Mullin 1976b), and it can be divided to three primary aspects: timing of acclimation, maximum cold hardiness capacity, and ability to resist rapid deacclimation or ability to reacclimate rapidly after unseasonable warm periods in late winter (Burke et al. 1976). Furthermore, evergreen plants may suffer from winter desiccation. Any one of these components may be the limiting factor in winter survival. Hardiness is further complicated by the fact that adjacent tissues of a plant may simultaneously have different hardiness levels (Weiser 1970, Sakai and Larcher 1987). Many economically important woody species possess an adequate ultimate midwinter hardiness level but sometimes the photoperiodically induced cold acclimation response might be improperly timed and plants cannot be successfully grown at high altitude or latitude (Hummel et al. 1982). For active hardening processes, the growing season should be considered more essential than the wintertime (Koski and Sievänen 1985).

1.4.1. Frost injury

If the plants are not properly acclimated they may be injured or killed even by a mild frost. Usually low temperature *per se* does not induce injury, but direct injury leading to the death of the cell is due to intracellular freezing, rapid temperature change or desiccation of the cells. The injury can occur during freezing, when frozen, during thawing or after thawing (Levitt 1980). Repeated freezing and thawing amplifies injuries (Burke et al. 1976). Evergreen leaves especially are susceptible to winter desiccation, that causes symptoms similar to freezing injuries. Due to desiccation the leaves first dehydrate and then turn necrotic, whereas frost primarily causes necrosis, which leads to decrease in water content (Sakai and Larcher 1987). Desiccation may occur within days or proceed during several winter months, when stomata are closed, but transpiration continues through the thin cuticle (Sakai and Larcher 1987). Dehydration during winter can lead to expansion of gas bubbles in the xylem (cavitation), which may cause severe injury to the xylem ray protoplasm (Ristic and Ashworth 1995). Frost injuries are seen as browning of various tissues.

The survival of an organ may depend on the survival of an adjacent organ. For example, lethal injury in leaf midribs and petioles may result in death of the whole leaves, which would greatly reduce the ornamental value of evergreen plants. Injury to inner bark also plays an important role in plant survival, damage to it may result in death of leaves, inflorescences and apical meristems (Holt and Pellett 1981). However, even if the leaves are killed, the plant may recover from the hardier vegetative buds, provided that the cambium survives (Sakai and Larcher 1987).

At the cellular level the symptoms of membrane injury are leakage of solutes, a water-soaked appearance, and the inability to reabsorb solutes and to regain turgor after the stress. If the damage is not severe, cells are capable of repairing it. Recovery occurs via activation of membrane pumps that transport leaked ions and solutes back into the cell (Palta et al. 1977, Palta 1990). Damage in proteins occurs as a loss of quaternary and tertiary structures of proteins, loss of enzyme activity, denaturation and aggregation of proteins (Levitt 1980). The cell may be able to repair partially denatured proteins (Palta et al. 1982). Large amounts of leaked K+ ions in the apoplast may be responsible for secondary injury (Palta et al. 1982).

1.4.2. Environmental control of cold acclimation

Cold acclimation of woody plants has been described as proceeding in three stages (Weiser 1970). The first stage is induced by critical photoperiod at adequate temperatures. It is energy consuming and depends on photosynthesis (McGuire and Flint 1962, Zehnder and Lanphear 1966, van den Driessche 1970, Weiser 1970). It involves storage of starch and lipids, decrease in water content and increasing desiccation tolerance (Levitt 1980, Sakai and Larcher 1987).

The second stage of hardening is induced by low temperature, 0 to 5°C (Weiser 1970). It is further triggered by frost (Sakai 1966, Weiser 1970), but not influenced by photoperiod (McGuire and Flint 1962, Zehnder and Lanphear 1966, Bigras et al. 1989). It involves considerable modifications of metabolism including structural changes and synthesis of proteins and membrane lipids. The permeability of protoplasm to water increases (Levitt 1980, Sakai and Larcher 1987, Hällgren and Öquist 1990) and low-molecular-weight compounds with

cryoprotectant activity accumulate in hardy plants (Guy 1990). Plants exposed to SD, but not to low temperature, only reach the first stage of acclimation (Weiser 1970).

In most temperate zone woody species cold acclimation is induced most effectively by a combination of SD and low temperature (Sakai 1966, van den Driessche 1970, Aronsson 1975, Smit-Spinks et al. 1985, Tremblay and Lalonde 1987, Puttonen and Arnott 1994). It is important to have SD prior to low temperature (Zehnder and Lanphear 1966, Irving and Lanphear 1967, van Huystee et al. 1967, Bervaes et al. 1978, Christersson 1978, Graham and Patterson 1982, Junttila and Kaurin 1990). Together SD and low temperature have an additive effect on hardiness (Christersson 1978, Greer 1983a), possibly involving also an interaction component (Bervaes et al. 1978). Chen and Li (1978) proposed that low temperature, SD and water stress trigger independent frost hardening mechanisms, and total hardiness would be a sum of these. Cold acclimation can also be induced by abscisic acid (ABA) or water stress (Graham and Patterson 1982, Thomashow 1999).

In Weiser's (1970) model, very low temperatures −30 to −50°C induce a third stage of hardiness. However, for willows (*Salix*) and poplars (*Populus* L.) hardening below −20°C was negligible (Sakai 1966).

1.4.3. Changes in metabolism during cold acclimation

During active growth in summer almost all metabolites are used for growth (Levitt 1980), but after growth cessation and during cold acclimation plants accumulate carbohydrates among other metabolites. Correlation between sugar content and cold hardiness has been reported in many woody species (Sakai 1966, Sakai and Yoshida 1968, Siminovitch 1981, Fisher and Höll 1991, Flinn and Ashworth 1995, Leborgne et al. 1995ab, Imanishi et al. 1998, Palonen 1999). Development of complete hardiness involves conversion of starch to sugars, which is triggered by low temperature (Sakai 1966, Siminovitch 1981), and has been observed also in evergreen leaves of *Nothofagus* Blume (Alberdi et al. 1989). Dehardening involves the conversion of sugars back to starch (Siminovitch 1981). Different sugars have a major role as cryoprotectants in increasing solute concentration and thus depressing the freezing point of cells, and furthermore, in stabilizing membranes (Levitt 1980, Guy 1990, Hinesley et al. 1992, Steponkus et al. 1993). Sucrose, fructose, raffinose and sorbitol are mentioned as primary protectants (Levitt 1980, Guy 1990, Leborgne et al. 1995b), but also other sugars and sugar derivatives, certain amino acids and polypeptides are involved (Santarius 1982). Different transport carbohydrates accumulate in various species (Levitt 1980, Flinn and Ashworth 1995, Leborgne et al. 1995a). Wright and Aung (1975) found that in mid-winter the predominant sugars in *Rhododendron* 'Sweetheart Supreme' and 'Hexe' were fructose, glucose, sucrose and lesser amounts of raffinose and maltose. Sugar content was higher in roots than in leaves, stems and buds.

The soluble protein content of plants increases during hardening, but there are also qualitative shifts, as cold acclimation includes changes in gene expression (Guy 1990, Thomashow 1999). The primary changes occur most probably at the transcriptional level (Weiser 1970, Mohapatra et al. 1987, Thomashow 1990). Some preexisting proteins disappear, others increase in amount, and cold-activated genes synthesize new ones (Li et al. 1965, Schneider 1965, Graham and Patterson 1982, Mohapatra et al. 1987, Thomashow 1999).

Lim et al. (1999) compared hardiness and dehydrin content in *R. catawbiense*, *R. fortunei* Lindl. and their hybrid 'Ceylon'. Dehydrins are hydrophilic, heat-stable proteins induced in plants in response to dehydrating stresses, such as salt, water or freezing stress. They accumulate in plant tissues during cold acclimation. 25-kDa-dehydrin level alone was a reasonably good predictor of cold hardiness in *R. maximum* and in cultivars 'Hawaii', 'Swansdown' and 'Pink Parasol', in which the increase in dehydrin was related to increasing hardiness with increasing age (Lim et al. 1999). Dehydrins could be used as both quantitative and qualitative markers for hardiness also in *R. catawbiense*.

As early as 1912, Maximov suggested that disruption of the plasma membrane is the primary cause of freezing injury (see Levitt 1980), and since then it has become evident that the maintenance of the structural integrity of the plasma membrane is a prerequisite for the survival of the cell (Palta 1990). It serves as a barrier between the cytosol and the extracellular space allowing water efflux during the freeze-thaw cycle (see Sakai and Larcher 1987, Steponkus et al. 1993). Typically the plasma membrane contains phospholipids, sterols and their derivatives, which include glucose. Glucose and fructose have a role also in protecting other cell membranes (Santarius 1982). The proportion of phospholipids and diunsaturated fatty acids increases during cold acclimation, as there is a need to increase fluidity of membranes at low temperatures (Graham and Patterson 1982, Sakai and Larcher 1987, Thomashow 1990, 1999, Steponkus et al. 1993).

Loubaresse and Dereuddre (1990) found that the levels of phospholipids and galactolipids increased in the roots of *Rhododendron* 'Jean Marie de Montaguë' during cold acclimation. Linolenic acid accumulated at the expense of linoleic acid, and the level of unsaturation increased. After a freezing stress, the levels of phospholipids and galactolipids decreased, but remained higher in cold acclimated than in non-acclimated roots (Loubaresse et al. 1991). As well, peroxidation was moderated in the acclimated roots. Injuries were accompanied by an accumulation of phosphatidic acid. Increased peroxide and malondialdehyde levels during freeze stress were possibly related to catabolism of polar lipids (Loubaresse et al. 1991).

1.4.4. Mechanisms for frost tolerance

Plant tissues can survive frost in different ways: 1) by avoiding intracellular freezing, but tolerating extracellular freezing and dehydration of cells, 2) by supercooling, which is typical for xylem rays and floral buds, or 3) by depressing the freezing point with antifreeze proteins or other cryoprotectants or by dehydration (Levitt 1980, Sakai and Larcher 1987). The most common way in which a winter-hardy plant cell survives subfreezing temperatures is by losing cellular water to extracellular ice, which leads to dehydration of the cells and cell solute concentration (George et al. 1982). At slow cooling rates the freezing occurs in equilibrium: a water potential gradient is established, and liquid water moves out of the cell to the extracellular space (Guy 1990). Extracellular freezing can lead to excessive dehydration and plasmolysis of cells (Levitt 1980). Rapid cooling often causes injury due to intracellular freezing, if the rate of the water diffusion from the cell interior to apoplast can not follow the rapid cooling rate (Levitt 1980). Weiser (1970) suggested that during freezing a point is reached when all readily available water has been frozen extracellularly and only "vital" or bound water remains in the cell. He proposed that intracellular ice forms when this water freezes. However, there is controversy about whether this bound water can freeze or not.

Supercooling of xylem ray parenchyma is a freeze avoidance mechanism, where cells can retain cellular water in a liquid phase at subfreezing temperatures. It can occur in tissues with small cells, little intercellular space and low water content (Sakai and Larcher 1987). Supercooling can only proceed to a certain steady level, which varies seasonally and after which intracellular freezing and death will occur (George and Burke 1977a, Levitt 1980). The freezing point can be observed as a low temperature exotherm (LTE) in differential thermal analysis (DTA) (Quamme et al. 1972, George et al. 1974a, Graham and Mullin 1976a, Ishikawa and Sakai 1981). In addition to DTA studies, the formation of intracellular ice crystals has been proved in a microscopic study (Ristic and Ashworth 1993), but supercooling of azalea xylem was not detectable in nuclear magnetic resonance (NMR) (Price et al. 1997). After ice nucleation, each ray or small groups of rays are nucleated independently and the whole ray freezes in a rapid manner (Burke et al. 1976, George and Burke 1977a, Hong and Sucoff 1980, Ristic and Ashworth 1994). No barriers for ice growth exist from cell to cell in the ray (George and Burke 1977a). Slow freezing results in a few large ice crystals, while fast freezing results in many small crystals, since rapid freezing does not permit water migration from cells (Burke et al. 1976). In frozen evergreen azalea stems ice was found to be widely distributed in small crystals throughout the xylem tissue (Lumis et al. 1972). When the water content was high, large ice masses caused splits, whereas dried twigs did not split and survived lower temperatures. The freezing point for fully acclimated xylem may be as low as –47°C, lower than the homogenous nucleation temperature (–40°C) for pure water (George et al. 1974a). The xylem rays of many woody species native to temperate regions supercool and the ultimate low winter temperatures limit the cultivation of these plants (Sakai and Weiser 1973, George et al. 1974a, Siminovitch 1981, Sakai 1982). Plants that supercool tend to have ring-porous xylem (George et al. 1974a) and are only moderately hardy (Ristic and Ashworth 1995).

In very hardy arctic and sub-arctic woody plants, such as *Betula*, *Populus* and *Salix* the xylem rays do not supercool, but their survival depends upon tolerating extracellular ice formation and cell dehydration (Sakai and Weiser 1973, George et al. 1982, Larcher 1982, Ashworth 1996). These species do not have LTEs (George et al. 1974a, Burke et al. 1976, Ristic and Ashworth 1994), and they can survive immersion in liquid nitrogen (–196°C) when fully cold acclimated (Sakai 1960).

In floral primordia of many species water is liquid even though ice crystals are present in adjacent bud axis and scales. Supercooling of these florets is different from that of xylem ray parenchyma (Ishikawa and Sakai 1981). At slow cooling rates, water migrates out of the flower bud resulting in the enhancement of supercooling and complementary ice accumulation in bud scales (Ashworth 1982, Ishikawa and Sakai 1982). The phenomenon is defined as extraorgan freezing (Ishikawa and Sakai 1981, 1982). Bud scales may also protect the flower primordia from external ice crystals (Chalker-Scott 1992).

1.4.5. Inheritance of cold hardiness

Cold hardiness is generally regarded as a quantitatively inherited trait requiring expression of many genes (see Stushnoff et al. 1985, Guy 1990, Bourne and Moore 1992). In many herbaceous and woody plants cold hardiness has been found to be inherited in a predominantly additive manner, the F_1 being intermediate to parents (Hummel et al. 1982, Timmis et al. 1991, Lim et al. 1999) or it is controlled by an additive-dominance system where hardiness is either a dominant or a recessive character (Guy 1990, Limin and Fowler 1993, Stone et al.

1993, Sutka 1994). Only a few, or a large number of genes with small effects may be involved (Mowry 1964, Toyao 1982, Stushnoff et al. 1985, Stone et al. 1993, Thomashow 1990, Teutonico et al. 1995).

The gene effects of *Rhododendron* cold hardiness were studied by Lim et al. (1998a, 1999). As the F_2 and backcross populations segregated for cold hardiness and had a continuous distribution pattern, the inheritance seems to be polygenic. The hardiness of the F_1 was uniform, and the mean hardiness of F_1 and F_2 populations were similar. The F_1 was closer to the hardier parent, and the two reciprocal backcross populations differed to some extent (Lim et al. 1998a). It is possible that as few as three genes with strong additive effects control variation in hardiness in *Rhododendron*, and one of these could be a 25-kDa-dehydrin coding gene (Lim et al. 1999). It was present in *R. catawbiense*, but not in the less hardy *R. fortunei*, and accumulated at an intermediate level in 'Ceylon', which was also intermediate in cold hardiness. The authors proposed a dominant or codominant inheritance of the dehydrin expression.

In some plants, such as blackberries (*Rubus*), dominance may play a role in determining cold hardiness response, and a single hardy parent can contribute hardiness comparable to that of two hardy parents (Bourne and Moore 1992). According to Cox (1991) *Rhododendron minus* var. *minus* Carolinianum group can pass its hardiness to the progeny even when the other parent is quite tender.

Cold hardiness in the non-acclimated state and cold acclimation ability are separate heritable traits, which are supposedly not genetically correlated (Stone et al. 1993, Teutonico et al. 1995). Several different mechanisms may be involved. For example, the direction of dominance may depend on the severity of the freezing conditions (Rohde and Pulham 1960, Eunus et al. 1962, see also Guy 1990, Thomashow 1990). Different allozymes in heterozygotes may have different temperature optima (Tigerstedt 1985). Thus a heterozygote would show greater tolerance or thermostability over a range of temperatures.

No maternal effect of cold acclimation has been noted in *Cornus sericea* L. (Hummel et al. 1982) and numerous other genera, whereas in some genera maternal effect has been found (see Stushnoff et al. 1985, Guy 1990), for example in Scots pine *Pinus sylvestris* L. (Nilsson 1988). Based on field survival of hybrid progenies Uosukainen and Tigerstedt (1988) identified a strong maternal effect of cold hardiness inherited from *R. brachycarpum* subsp. *tigerstedtii*. However, there are contradictory statements about the possibility of maternal effect in *Rhododendron* or other woody species.

1.5. Cold hardiness of *Rhododendron*

In horticultural literature and nursery catalogs *Rhododendron* cold hardiness is conventionally expressed using floral bud survival in ideal conditions as a criterion. However, cold hardiness in winter is affected by many environmental factors such as cooling rate, humidity, snow coverage and wind, that may also enhance cold tolerance by dehydration (Ishikawa and Sakai 1981). Cooling rate may affect the lethal temperature of florets, leaves and stems by several degrees, as at rapid cooling rates the organs freeze at higher temperatures than at slow cooling rates (George et al. 1974b, Kaku et al. 1980, Ishikawa and Sakai 1981, Anisko and Lindstrom 1996a). According to Havis (1964) cooling rate did not affect the frost survival of thoroughly hardened rhododendron leaves, whereas Anisko and Lindstrom (1996a)

found that the influence of increasing cooling rate was more pronounced when hardening proceeded. The moisture content did not have a significant effect on frost survival at slow cooling rates (Havis 1964).

1.5.1. Hardiness of various organs

The flower bud is the most susceptible overwintering organ in many *Rhododendron* species and cultivars (Iwaya-Inoue and Kaku 1983, Sakai et al. 1986, Väinölä et al. 1997). Azalea flower buds can be hardy to –10°C in August (Graham and Mullin 1976b), and attain maximum hardiness in mid-winter. Several species and hybrids may survive –30°C (Pellett and Holt 1981, Sakai et al. 1986), and some of them tolerate –34 to –38, occasionally –40°C (Pellett et al. 1986, Uosukainen and Tigerstedt 1988, Väinölä et al. 1997). The frost survival mechanism of *Rhododendron* flower buds will be discussed below.

Based on observations on 101 *Rhododendron* genotypes Sakai et al. (1986) confirmed that xylem was less hardy than vegetative buds, leaves, and stem cortex or cambium, which could at best tolerate –60°C. The xylem may tolerate –40°C. Leaves, which may supercool to –7 to –13°C, vegetative buds and inner bark can tolerate ice, and thus their freezing points and exotherms are not related to cold hardiness (Graham and Mullin 1976a, Kaku 1993, Nilsen 1993). The freezing point of leaves is affected by desiccating winds (Havis 1964). Holt and Pellett (1981) found stem xylem and leaf interveins more cold hardy than leaf midrib and petiole or cambium, phloem and cortex.

Iwaya-Inoue and Kaku (1983) could not generalize the relative hardiness between xylem and bark in 24 native Japanese *Rhododendron* species. The contradictory results on the relative hardiness of xylem and bark may be due to different rates of cold acclimation in various tissues, as cambial growth ceases later than elongation growth (Wareing 1956, Håbjørg 1972, Heide 1974) and thus bark tissues acclimate later than xylem (Burke et al. 1976, Holt and Pellett 1981). The pith of one-year-old twigs of *R. japonicum* (A. Gray) Suring. [*R. molle* ssp. *japonicum* (A. Gray) Kron] remained alive after maturation and supercooled to –21°C before freezing (Price et al. 1997). Pith is the least hardy part of the twig (Lumis et al. 1972, Väinölä et al. 1997). According to Lumis et al. (1972) pith cells are not long lived and are expected to die during the first winter. They were killed at 2°C above floret killing temperature.

Bark split of the lower stem in fall and early winter is common in evergreen azaleas as the lower stems are less hardy than upper stems (Alexander and Havis 1980a, Anisko and Lindstrom 1996b). This problem does not apply to deciduous azaleas (Alexander and Havis 1980a). Exposing evergreen azalea 'Springtime' roots to low temperature enhanced the hardening of all plant parts, but the effect was seen most clearly in lower stems and roots, which were equally hardy (Alexander and Havis 1980b). Upper branches of both evergreen ('Mother's Day') and deciduous ('Homebush') azaleas acclimated to a greater degree than roots, and the lower stems of the deciduous ones were able to acclimate at a faster rate than roots (Alexander and Havis 1980a). In many plants roots with high water content are less hardy than aerial plant parts (Burke et al. 1976), but the roots of container grown plants exposed to low temperatures can harden to a greater extent than those in the ground. Havis (1976) found the roots of container grown *R. carolinianum* Rehder [*R. minus* var. *minus* Carolinianum group] and *R. catawbiense* able to tolerate –17.8 and those of PJM hybrids –23.3°C. The effect of photoperiod on hardening of roots has not been studied in *Rhododendron*, but the roots of some other woody species did not harden by exposure to SD,

but the roots of some other woody species did not harden by exposure to SD, but only due to low temperature (Smit-Spinks et al. 1985, Tremblay and Lalonde 1987, Bigras and D'Aoust 1993, Ryyppö et al. 1998).

The rate of cold acclimation, potential mid-winter hardiness and the rate of deacclimation are not necessarily related (Kaku et al. 1983, Iwaya-Inoue and Kaku 1986, Lim et al. 1998a). The *Rhododendron* taxa with the greatest midwinter hardiness do not necessarily possess superior hardiness earlier and later in the dormant period (Sakai et al. 1986), and cultivars with more hardy leaves or stems may not have the most hardy flower buds (Pellett and Holt 1981). The largest differences in hardiness among tissues within a plant were found in the most hardy *Rhododendron* species and cultivars (Sakai et al. 1986), whereas Lim et al. (1998ab) found the relative frost tolerance of leaves and flower buds, and on the other hand, leaves and vegetative buds similar in five *Rhododendron* cultivars. As well, according to Spethmann et al. (1998) flower bud, leaf and cambium survival of *Rhododendron* cultivars correlate with each other.

1.5.2. Irrigation and cold hardiness

Anisko and Lindstrom (1995, 1996abcd) studied the effects of reduced watering on the hardiness of evergreen rhododendrons and azaleas. If applied during the active growing period, reduced watering did not stimulate cold acclimation of leaves and current year twigs (Anisko and Lindstrom 1995), but a 3-6 weeks water stress in early autumn increased frost hardiness by 0.5 to 5°C (Anisko and Lindstrom 1995, 1996bcd). The drought induced hardiness gradually decreased in three weeks after a return to normal watering. The effects of drought may change if applied for two years. Severe water stress increased hardiness of 'Catawbiense Boursault' leaves and stems compared with well watered plants in the first winter, but decreased it in the subsequent winter, while moderate continuous water stress did not increase the cold hardiness during the first winter, but increased it in the second winter (Anisko and Lindstrom 1996c). Leaves and stems of the plants under a continuous dry regime were less hardy than those under a medium or wet watering regime (Anisko and Lindstrom 1996a). Continuous sufficient watering, especially combined with high temperature, can delay acclimation of the upper plant parts (Anisko and Lindstrom 1995). Anisko and Lindstrom (1996d) suggest that primary mechanism of the drought-induced increase in cold hardiness involves an increase of cell wall rigidity, which increases the resistance to freeze dehydration.

1.5.3. Adaptive mechanisms for frost survival of *Rhododendron*

1.5.3.1. Flower buds

Rhododendron flower development consists of two growth stages separated by a resting stage in winter. The flower buds start to develop in late summer, when all organs are formed, and pistils and stamens can be easily recognized. Before the rest period the buds accumulate storage-protein bodies and amyloplasts, that are divided among different tissues and are used in the following spring for cell elongation growth (Schneider 1972). The flower primordia in dormant *Rhododendron* flower buds, as well as the living cells in the xylem, survive low temperatures by deep supercooling and avoiding ice crystallization (George et al. 1974b, Graham and Mullin 1976a, George and Burke 1977b, Ishikawa and Sakai 1981).

The prevention of ice growth into floral tissues has been studied most thoroughly in azalea (Ishikawa and Sakai 1982, Chalker-Scott 1992) and *Prunus* L. (Ashworth 1982, 1984, Quamme et al. 1995). The mechanism seems to be similar in the two. The point of attachment of the flower primordium to the stem axis is critical. During dormancy vascular tissues in the bud axis and primordium are not yet differentiated, but there are only vascular traces consisting of procambium cells (Ashworth 1982, Ishikawa and Sakai 1982, Quamme et al. 1995). Xylem continuity is established during the time the capacity to supercool is lost (Ashworth 1984). Chalker-Scott (1992) found three possible barrier locations for ice growth in azalea bud tissues: 1) the cell layers between bud scales and axis, 2) the junction of stem to flower bud, and 3) the pedicel tissues of each floret. All these tissues are lignified and suberized and thus recalcitrant to freezing. Wounding of the barrier mechanically or by high temperature inhibits supercooling (Ashworth 1982, Chalker-Scott 1992).

The supercooling ability varies seasonally (Kaku et al. 1980, Ishikawa and Sakai 1981, Ashworth 1984), and the hardiness of florets is highly correlated with moisture content (Graham and Mullin 1976b, Ishikawa and Sakai 1981, Kaku et al. 1981, Iwaya-Inoue and Kaku 1986). Avoidance of freezing injury depends on the ability to lose water rapidly at a falling temperature (Graham and Mullin 1976b, Ishikawa and Sakai 1981), or on low water content *per se* (Kaku et al. 1981). Water content of axes and florets decrease during freezing (Kaku et al. 1980) and due to this the florets shrink during cooling, as was detected in NMR image (Price et al. 1997) and as LTE size in DTA (Ishikawa and Sakai 1981).

Unhardened flower buds freeze as whole units and no LTEs can be detected (Kaku et al. 1980, Ishikawa and Sakai 1981). The percentage of florets with LTEs increases during cold acclimation (Chalker-Scott 1992) and in winter the number of primordia may correspond exactly to the number of exotherms, although they may also overlap (George et al. 1974b, Graham and Mullin 1976a, Kaku et al. 1980). The range of exotherms of *Rhododendron* florets has been found to be narrow in midwinter, and in hardy plants, while a wide range was typical for non-acclimated or less hardy plants (Kaku et al. 1981, Iwaya-Inoue and Kaku 1983). However, the buds containing several florets had a broad exotherm span (Iwaya-Inoue and Kaku 1983). Graham and Mullin (1976a) found 6.5 to 10°C differences within a single bud. A wide temperature range for killed florets of deciduous azaleas was detected also visually (Pellett et al. 1991). The internal bud morphology, i.e. the order of scales and florets in the bud did not seem to affect the LTE width (Iwaya-Inoue and Kaku 1983), neither did the position of the floret within the bud relate to hardiness (Chalker-Scott 1992). Dead florets retain their supercooling ability, exhibit exotherms and freeze at the same temperatures as living primordia (George et al. 1974b, Graham and Mullin 1976b, Ishikawa and Sakai 1981, Chalker-Scott 1992).

1.5.3.2. Xylem and foliage

Dehydration during winter can lead to expansion of gas bubbles in the xylem (cavitation), which may cause severe injury to the xylem ray protoplasm (Ristic and Ashworth 1995). In *Rhododendron* the xylem vessels are small, which serves to minimize winter cavitation. Under a tree canopy, in a shaded environment, the vessels and the functional cross-sectional area of xylem in *R. maximum* have been found to be smaller and the plants better protected against loss of hydraulic conductivity than under high light environments (Lipp and Nilsen 1997).

Low stomatal conductance and low maximal net photosynthetic rate characteristic for *Rhododendron* may be adaptations to winter drought (Ranney et al. 1995). However, as the net photosynthesis decreases during hardening, the leaves become sensitive to photoinhibition, as was noted in *R. ferrugineum* (Bauer et al. 1994). Normally the stomata of *Rhododendron* are closed in winter, and thus photosynthesis is prevented (Nilsen 1985). Stomata may become active during infrequent periods in winter, if air and leaf temperatures rise to 10°C (Lipp and Nilsen 1997). Leaf temperature tends to be slightly higher than that of air, and the leaves in direct sunlight may be several degrees above 0°C, when air temperature is well below 0°C (Havis 1964, Neuner et al. 1999b). During warm winter days the difference between leaves and air can be 10°C (Nilsen 1985), and a leaf temperature of 16°C has been measured in *R. ferrugineum*, while the soil was still frozen (Larcher and Siegwolf 1985). After activation of metabolic processes by sunny weather, the stomata open and *Rhododendron* leaves can become dehydrated to such an extent that irreversible disturbances on chloroplast functions occur within days (Larcher and Siegwolf 1985).

Due to the absence of leaf canopy, irradiation in natural *Rhododendron* forest habitats is higher in winter than in summer. This causes a high risk of photoinhibition (Nilsen 1987). Photoinhibition of photosynthesis is caused by excess of excitation energy, when more photons are absorbed than are required to drive photosynthesis, often together with chilling or freezing temperatures even in moderate light (Krause 1988). Inactivation of the electron transport system of photosystem II (PSII) leads to reduction in the maximum quantum yields for CO_2 uptake and O_2 evolution, and retardation of photosynthesis (Öquist et al. 1987, Krause 1988, Nilsen et al. 1988). Genetically shade-adaptive plants – such as *Rhododendron* – with low capacity for carbon metabolism exhibit stronger photoinhibition than sun-adapted plants (Krause 1988). Plants can recover by *de novo* synthesis of photodamaged proteins in chloroplasts within minutes or hours after a move to low or moderate light, but prolonged exposure to high light and low temperature may cause pigment bleaching (Sakai and Larcher 1987, Öquist et al. 1987, Krause 1988). Desiccation induced by photoinhibition in *R. ferrugineum* diminished with the onset of complete snow cover, which protects the plants and promotes the recovery of PSII efficiency and the restoration of water relations. Recovery from photoinhibition required both protection from high irradiance and exposure to temperatures close to but above 0°C (Neuner et al. 1999a).

Furthermore, high irradiation hastens senescence. The chlorophyll content of *Rhododendron* leaves may remain high for six years under an evergreen canopy being highest in two- or three-year-old leaves, but it decreases rapidly during aging in exposed environments and in horizontal leaves (Gholz 1978, Nilsen and Bao 1987, Bao and Nilsen 1988, Nilsen et al. 1988). When most of the leaves of *R. maximum* under an evergreen canopy persisted for five seasons, under a deciduous canopy they abscised a year earlier and in the open most leaves abscised after two summers (Nilsen 1986). A similar trend was found in *R. macrophyllum* habitats (Gholz 1978).

It was noted already a hundred years ago that *Rhododendron* leaves curl at freezing temperatures (Harshberger 1899). Curling occurs in 10-20 minutes in response to ambient temperature below –2 to –5°C, a few degrees above the leaf freezing point and irrespective of irradiation (Fukuda 1933, Nilsen 1985, 1987, 1991). The lower the temperature the more intense is the curling (Fukuda 1933, Nilsen 1987, 1991). In summer curling is induced by water stress, but maximum curling at turgor loss point is only 30-45% of that induced by frost. Thus curling is only partially dependent on low leaf water potential (Nilsen 1987).

Rhododendron species have different associations between leaf curling, leaf water deficit and leaf temperature (Nilsen 1991, 1993). The intensity of curling was related to cold hardiness ratings of species, but not to leaf area or other morphological measurements (Nilsen 1993). Although the very hardy hybrids had strong curling and the sensitive hybrids very little curling, the hardiness ratings of hybrids was not consistently related to the curling rate (Nilsen 1993). In species the genes for leaf curling seem to be associated with those for cold hardiness, but since the association is lost in hybrids, these genes seem to be unlinked (Nilsen 1993). For example *R. catawbiense* and *R. minus* (Nilsen 1991), *R. maximum* (Harshberger 1899, Nilsen 1991), *R. micrathum* Turcz. (Fukuda 1933) and *R. brachycarpum* (personal observation) belong to curling sensitive species. The physiological cause of leaf curling is not yet well understood (Nilsen 1992).

Low temperature also affects the leaf angle of *Rhododendron*, but in a different manner than curling (Nilsen 1985). The angle is controlled by leaf water potential, temperature and light (Nilsen 1985, 1991, 1992). The drooping response of species to cold seems to be dependent on the length of the petiole, the species with long petioles having stronger angle movements than species with short petioles (Nilsen 1991). Since the petiole is not necessarily in equilibrium with the water deficit of the leaf blade, and its cuticle is minimal, it dehydrates easily (Nilsen 1987). As a consequence, leaves are completely pendent when the petiole cells lose turgor pressure. In response to rising temperatures during warm winter days the leaf first uncurls, then rises to a more horizontal position (Nilsen 1992). The intensity of these two leaf movements is correlated with cold tolerance (Nilsen 1991).

According to Nilsen (1992) two of the six possible adaptive benefits of thermotropic leaf movements are relevant. Firstly, leaf movements reduce the freeze-damage susceptible leaf surface exposed to cold night sky or daytime sun (Bao and Nilsen 1988, Nilsen 1991). At night curled and pendent leaves have higher temperature than uncurled and horizontal ones, and during daytime leaf position could reduce the speed of leaf thaw during sun flecks that cause rapid changes in leaf temperature (Havis 1964, Bao and Nilsen 1988). Secondly, leaf movements protect the leaves and their chloroplast membranes against photoinhibition. Without leaf movements the leaves would be injured during bright, cold winter days, and as a consequence, the net photosynthetic rates in the following summer would be reduced and leaf mortality would increase (Bao and Nilsen 1988). Nilsen (1992) concluded that changes in leaf angle protect against photoinhibition, and leaf curling protects against freezing damage by rapid thaw.

Rhododendron may have the ability to acclimate to extreme irradiance conditions. Dunning et al. (1994) found that UV-B treatment increased cold hardiness of 'English Roseum' leaves by 3°C. The treated leaves were smaller and more reddened than the untreated leaves, and the levels of water-soluble phenolics were doubled. The authors suggest that phenolic compounds synthesized in response to one stress may protect the plant against other stresses.

2. AIMS OF THE STUDY

The main objective of the study was to facilitate breeding of *Rhododendron* by investigating factors which affect cold hardiness of rhododendron and azalea. More specifically, the following objectives were included:

1) to compare the relative cold hardiness of stem and flower bud tissues of deciduous azaleas throughout the dormant period (I)
2) to investigate how different growing season temperatures and photoperiods affect growth (II) and cold hardening (III) of rhododendron cultivars
3) to compare the cold acclimation potential and hardiness of diploid rhododendrons and the corresponding autotetraploids, which have the same chromosomes and genes, but in a doubled form (IV, V)
4) to compare the effectiveness of visual evaluations of injury with electrolyte leakage measurements as methods of determining cold hardiness (I, III, IV)
5) to assess whether impedance spectroscopy can be used to study cold hardiness of rhododendron leaves, and whether the changes in equivalent circuit parameters during acclimation can be used to predict frost hardiness without controlled freezing tests (V).

3. MATERIALS AND METHODS

Materials and methods are described here only briefly. More detailed information can be found in the original publications (I-V).

3.1. Plant materials, growing conditions and acclimation procedures

Evergreen rhododendrons (II-V) and deciduous azaleas (I, II) were used in the studies. Two elepidote (subgenus *Hymenanthes*) cultivars *R.* 'Helsinki University' and *R.* 'Pohjola´s Daughter' represented the rhododendron breeding program of the University of Helsinki (II, III). *R.* 'Cunningham´s White' is an old English cultivar from 1830 and its tetraploid derivative CW4 was created for these experiments (Väinölä 2000) (IV, V). These two were produced *in vitro* at the Department of Plant Biology, University of Helsinki. The lepidote (subgenus *Rhododendron*) cultivars *R.* 'PJM' (1939) and *R.* 'Northern Starburst' (1997) are newer releases from the United States (IV, V). Deciduous azaleas represented the breeding programs of the University of Minnesota (I) and the University of Helsinki (II).

Mature plants of five deciduous Lights azalea clones 'White Lights', 'Spicy Lights', 'Mandarin Lights', 570091 and 800104 were growing in a field plot at the Minnesota Landscape Arboretum in Chanhassen, Minnesota (44°50' N), where the minimum air temperature during the winter 1992-93 was -29°C. (I)

One-year-old clonally propagated evergreen rhododendron cultivars, 'Helsinki University' and 'Pohjola´s Daughter' (II, III) and five-year-old full-sib seedlings of deciduous azaleas, *R. canadense* (L.) Torr. and #89132 (II), were grown for 112 days under either a short day (SD, 14 h) or a long day (LD, 20 h) photoperiod combined with temperatures of 15 and 24°C. Additionally, these cultivars were compared for daylength extension at 24°C/LD under two different irradiation treatments (incandescent bulbs and fluorescent tubes) (II).

After 112 days in selected growing season conditions 'Helsinki University' and 'Pohjola´s Daughter' were hardened at a lowering temperature regime at fortnightly intervals: +9, +5, +1 and –2°C. The photoperiod was controlled as shown in Fig. 2B in III. Additionally, half of the plants grown in LD were submitted to a 20-h photoperiod during the first 4 weeks of acclimation and thereafter at +1 and –2°C like the SD plants. (III)

Hardiness of diploid and tetraploid plants was compared. Experiments were done with two lepidote rhododendron clones, a diploid 'PJM' and its autotetraploid derivative 'Northern Starburst', and two elepidote clones, a diploid (CW2) and a tetraploid (CW4) form of 'Cunningham's White'. After summer in a greenhouse the temperature was allowed to decrease and in October it was maintained at 5-10 °C. During the beginning of November, plants were moved to a cold acclimation regime in phytotrons. The acclimation regime consisted of three consecutive weeks at each of the temperatures +5, +1 and –2°C. The photoperiod was kept at six hours. (IV, V)

3.2. Growth and anatomy

At the end of the growing season in different photo- and thermoperiod combinations the following growth variables were observed: the total plant height, the number of consecutive

new flushes, the length of each flush, and the number of leaves on the flushes. On each plant the length of the flushes and the number of leaves on them were measured on the leader branch (evergreens) or on a well-formed branch in the middle of the bush (azaleas). In addition, the total number of flower buds on each azalea was recorded. (II)

To study the cell size, microscope slides of leaves and stem sections were prepared. The thickness of the leaves was measured at the interveinal area after freezing and thawing. (IV)

3.3. Determination of cold hardiness

The hardiness of the current year's shoots and flower buds of azaleas (I) was studied six times during the winter 1992-93 from November until April. The samples were frozen in a programmable freezer in polyethylene bags from the prevailing outdoor temperature at a rate of 5.6°C h^{-1}, removed from the freezer at 2-3°C temperature intervals and thawed at 2°C.

The hardiness of the evergreen leaves (III-V) as well as shoots and flower buds (IV) was assessed before each decrease in the acclimation temperature and at the end of the experiments. Samples were frozen either

1) buried in wet sand in aluminum boxes from 0°C at the cooling rate of 3.3°C h^{-1} down to –18°C, thereafter at 10°C h^{-1}, taken out at 2-3°C temperature intervals, thawed at 1.5°C overnight and thereafter kept for 3-4 h at room temperature (III), or
2) in polyethylene bags from 5°C at a rate of 5°C h^{-1} (IV, V). The target temperature was held for four hours, and the temperature was then raised to 5°C at the rate of 5°C h^{-1}. Control samples in all experiments were kept unfrozen.

The injury on leaves and stem sections was assessed visually and with electrolyte leakage (EL) tests (I, III, IV), or based on data on extracellular resistance r_e and relaxation time τ of the analysis by impedance spectroscopy (IS) (Repo 1994, Repo et al. 1994) of the freeze-stressed leaves (V). Relative electrolyte leakage (REL) was calculated as: (initial ion leakage/final ion leakage)*100%. Flower bud hardiness was studied visually (I) or with differential thermal analysis (IV). In addition the hardening of leaves was estimated from impedance parameters r_e and τ of the unfrozen samples (V).

In paper I cold hardiness is presented in the LST (lowest surviving temperature) format, where the hardiness estimate is the minimum temperature at which >50% of the florets or the stem samples survived. In papers III and IV the data from visual observations are presented as 95% confidence intervals along with mean values of the estimate. In paper V the estimates are given with standard errors.

3.4. Statistical analyses

Statistical analyses were done using the SAS statistical package (SAS Institute Inc. 1989). The frequencies of flushes of new growth were analyzed with Fisher’s exact test using PROC FREQ. The growth parameters were analyzed either using ANOVA and Tukey's test (PROC GLM) to separate the significantly different means, or if the data were not normally distributed, with a non-parametric Cochran-Mantel-Haenszel -statistic (PROC FREQ) after logarithmic transformation of the data. Since the cultivars differed in their growth pattern, the data were in the main analyzed separately for each cultivar. (II)

To determine the lowest surviving temperature in paper I, the EL data were analyzed with ANOVA and significantly different means were separated using the Student-Newman-Keuls multiple range test. The lowest temperature at which average specific conductance did not differ significantly ($p < 0.05$) from the control was used as LST. (I)

ANOVA (PROC GLM) was used also in analyzing the leaf thickness data and the data from the exotherm analysis (IV) as well as in predicting frost hardiness without freezing tests from the impedance parameters of unfrozen leaves (V). The means were compared using Tukey's test.

To determine cold hardiness a sigmoid function (PROC NLIN) was fitted to the data of the electrolyte leakage tests (III, IV) and parameters r_e and τ of the impedance analysis (V) across freezing temperature treatments for each clone and freezing date combination using the following equation:

$$Y = a + (b - a)/(1 + e^{c(d-T)}) \quad (1)$$

where Y is REL, r_e or τ, a and b are the asymptotes of the function, c is the slope at the inflection point d, and T is the treatment temperature. The temperature (d) at the inflection point was used as an estimate of cold hardiness.

The visual assessments of freezing damage were analyzed with logit models (Collett 1991). The equation used was:

$$\text{logit}\,[\pi(T)] = \ln\{\pi(T):[1-\pi(T)]\} = \alpha + \beta_1 T + \beta_2 \ldots + \beta_n + \gamma \quad (2)$$

where π is the probability of damage at the freezing temperature T, α is the intercept, the parameter vectors β describe the magnitude of the effects of freezing temperature, cultivar, growing season and acclimation (freezing test date) and γ describes the magnitude of the effects of the interactions. The parameter vectors had two to six components depending on the number of cultivars, growing season and acclimation treatments. The model parameters were estimated using PROC GENMOD (SAS Institute Inc. 1993) (III, IV). Logit models also allow estimation (with confidence intervals) of lethal temperature type quantities which indicate the temperature causing a certain (often 50%) risk of damage or death. As logit models can only be used with binary responses (e.g. damaged/not damaged), the initial five point visual damage (VD) score was reduced to a binary scale. In paper III this was done cumulatively in three different ways resulting in visual damage responses VD_{10}, VD_{50} and VD_{90}, where the leaf was rated damaged when at least 10%, 50% or 90% of the surface was brown, respectively. When the 95% confidence levels overlapped the hardiness of the plants did not differ significantly at $p<0.05$. The significance of the parameters was checked with a Wald test. To compare the visual damage scores with electrolyte leakage, the estimated $(VD_{10}T)_{50}$ (temperature causing 50% probability of 10% visual damage), $(VD_{50}T)_{50}$ and $(VD_{90}T)_{50}$ were correlated with LT_{50}, i.e. d of EL test.

4. RESULTS AND DISCUSSION

4.1. Cold hardiness of organs during winter (I)

Stems of Lights azaleas were usually hardier than the flower buds of the same plants. In mid-November the hardiness of the stems ranged from –25 to –34°C. Florets acclimated later than stems. By mid-November they had hardened to –22°C in all five clones, and were thus 3 to 15°C less hardy than stems. The flower bud hardiness of the clones generally followed the same rank order throughout the winter, but some inconsistency was observed among the stems. The maximum hardiness was attained in January, when stems of 'White Lights' and 'Mandarin Lights' and florets of 'Spicy Lights' withstood –40°C. That was the only case when florets were hardier than stems. The maximum midwinter hardiness of the florets of the other clones was 2 to 4°C less than that of the corresponding stems. A similar relationship has been reported for other *Rhododendron* species and cultivars (Iwaya-Inoue and Kaku 1983, Sakai et al. 1986), as well as for other woody genera (Proebsting 1970, Cappiello and Dunham 1994).

The florets of azaleas started to deacclimate earlier in the spring than the stems. From January till March florets deacclimated by 4 to 8°C, while little or no loss of hardiness was detected in stems. The days were very warm in early March, but there were frosts during the nights and also the maximum temperature returned to sub-freezing level prior to March sampling, when the florets were still hardy to –25 to –30°C. Even though the nights were still very cold, the florets did not reacclimate to the mid-winter level. Stems deacclimated later than florets in response to rising temperature and frostless nights, but were still ca. 12°C more hardy than florets in April. Both stems and florets deacclimated substantially and at similar rates from March till April.

Flower bud hardiness of azaleas has been found to respond to fluctuating temperatures even in mid-winter (Graham and Mullin 1976b). Dehardening of *Rhododendron* in spring can occur in response to a short exposure to high temperature or to a longer exposure to low, but above freezing temperature (Kaku et al. 1983). Pellett et al. (1991) reported that flower buds of deciduous azaleas dehardened rapidly in midwinter in response to increasing (0 to –2°C) mean daily temperatures, but that vegetative tissues may deacclimate as well. *Cornus stolonifera* Michx. stems deacclimated when minimum air temperature remained above freezing for several days (van Huystee et al. 1967). The hardiness of *R. ferrugineum* in spring seems to be best explained by monthly minimum mean temperature (Neuner et al. 1999b). Its leaves hardened and dehardened rapidly in response to temperature. However, the plants were able to reharden, although at a slow rate, despite the high daytime temperature (19°C), if there was frost at nights (Neuner et al. 1999b). According to Ishikawa and Sakai (1981) two cold days are sufficient for rehardening of rhododendrons, but according to Neuner et al. (1999b) rehardening proceeds slowly during the first three days with night frosts, and rapidly thereafter.

The differential hardening rate of azalea flower buds and stems may relate to their freezing avoidance mechanisms. Xylem ray parenchyma of *Rhododendron* supercool to avoid ice formation (Sakai et al. 1986), but the florets undergo a combination of deep supercooling and extraorgan freezing, which involves migration of cellular water between the florets and sinks (scales) in response to changing temperature (Ishikawa and Sakai 1982). The absence of water migration might account for the ability to avoid deacclimation in the xylem ray parenchyma.

4.2. Effects of growing season on growth and cold acclimation (II, III)

Photoperiod and temperature during the growing season affected both growth and cold hardiness of *Rhododendron*. Elongation growth occurred in flushes. The number of flushes varied from one to three, increasing with increasing photoperiod and temperature in the two evergreen cultivars and *R. canadense*; azalea #89132 made only one flush in all treatments. Thus the plants grew taller in LD than in SD. In native habitats *Rhododendron* plants usually make only one flush, occasionally two flushes a year (Lipscomb and Nilsen 1990). The photoperiod has a very strong effect on the number of flushes also in *Acer* L. (Olmsted 1951) and *Quercus* L. (Collin et al. 1996). The present study indicates that the photoperiodic effect is further modified by high growing temperature, as the 24°C/LD treatment contributed to a remarkable increase in the flush number. 'Pohjola's Daughter' continued growth regardless of the light level at 24°C/LD, and almost half of the plants made three flushes also in 24°C/SD. However, these third shoots were all very short. Interactions of daylength with the lower temperature or of the temperature with SD were less obvious or absent.

Photoperiod also had a strong effect on the elongation growth and leaf production of the two evergreen cultivars. LD contributed to longer flushes and more leaves than SD. According to Nilsen (1986) higher light level in a native habitat enhanced the growth of *R. maximum* similarly. Additionally, florist azaleas produced longer shoots and a larger number of leaves per shoot in LD than in SD (Pettersen 1972). In the present study, lower temperature promoted the length of the first flush, but the further flushes grew taller at 24°C or were not affected by the temperature. The number of leaves per shoot in the first flush did not differ significantly between treatments, indicating that this character was predetermined by conditions during bud development. On further flushes more leaves were produced at 24°C than at 15°C.

Generally, photoperiod and temperature had little effect on vegetative growth of azaleas and the flower bud production of *R. canadense*. Azalea #89132 formed more flower buds at 15°C than at 24°C, and at 24°C/LD it produced more flower buds when the supplementary light was sufficient for photosynthesis compared with the low irradiance photoperiod extension. The azaleas were already five years old, whereas the evergreen rhododendrons were only one year old.

In this experiment the temperature was held constant, which would not occur in nature. The alternating day and night temperatures might alter the growth pattern compared with constant temperature. In field experiments at 56°N the same rhododendron cultivars made only one flush per year, whereas some plants produced two flushes at 65°N in a very warm summer 1999 (A. Väinölä, unpublished). In previous years the low temperature of the northern location prevented further flushes.

In phytotrons, photoperiod and temperature applied during the growing season affected the development of cold hardiness of the evergreen cultivars, but they differed in their responses. 'Pohjola´s Daughter' benefited from SD as well as from high temperature, while 'Helsinki University' attained better hardiness at a cool growing season temperature, and was less sensitive to photoperiod.

After the growing season and initial hardening at 9°C the hardiest 'Pohjola's Daughter' and 'Helsinki University' plants were those grown at 15°C/SD (Table 1, Fig. 4 in III). The ones grown at 24°C/LD were the least hardy. At the end of the experiment the 'Pohjola´s Daugh-

ter' plants could be divided into four distinct hardiness groups according to the preceding growing season temperatures and photoperiods. The 24°C/SD growing season treatment contributed to the best hardiness. The plants grown at 15°C had made two flushes regardless of photoperiod; two or three flushes were produced at 24°C/SD and three flushes at 24°C/LD. The duration of growth as measured by the number of subsequent flushes did not correlate with the hardiness of 'Pohjola´s Daughter'.

The 'Helsinki University' plants grown at 15°C/SD were hardier than the plants from any other growing treatment throughout the experiment. Thus by the end of the experiment, the lower growing temperature had resulted in better hardiness than the higher one. Moreover, SD contributed to better hardiness than LD at both temperatures. The hardiest plants acclimated only after transfer to –2°C. The rank order of the treatments in inducing hardiness in 'Helsinki University' was the same as inducing growth cessation. The hardiest plants were the ones with only one flush grown at 15°C/SD. According to Levitt (1980) after growth cessation all photosynthetic assimilates can be used for the production of storage carbohydrates and for proper cold acclimation. Plants grown at 15°C/LD ceased growth after making one or two flushes and ranked second in hardiness. In the treatments of 24°C/SD and 24°C/LD the plants had made two and two or three flushes, respectively.

The effects of growing season conditions on hardening processes have been studied only a little. Aronsson (1975) found that Scots pine (*Pinus sylvestris*) grown in LD attained better hardiness if the temperature during growth was low, but hardiness of Norway spruce (*Picea abies* (L.) Karsten) was not affected by temperature. Similarly in Scots pine seedlings, better

Table 1. Cold hardiness estimates (°C) with 95% confidence intervals of 'Helsinki University' and 'Pohjola's Daughter' grown at different thermo- and photoperiods assessed visually and with an electrolytic leakage test (EL) at various stages of acclimation.

Cultivar	*Grown at*	*Test*	*Acclimated at*			
			9°C	*5°C*	*1°C*	*-2°C*
'Helsinki University'	15C/14h	visual	-16.5±3.3	-16.5±2.5	-15.5±2.0	-19.7±0.8
		EL	-15.0±1.7	-10.5±8.1	--[1]	-17.7±5.6
	15C/20h	visual	-10.5±2.5	-8.5±1.9	-13.5±1.8	-15.0±1.1
		EL	--[1]	-11.7±2.0	-11.1±9.7	-12.5±4.8
	24C/14h	visual	-9.4±4.0	-10.5±3.2	-13.6±2.8	-11.5±1.4
		EL	-10.2±4.7	-14.5±3.0	-12.7±4.8	-11.1±0.9
	24C/20h	visual	-5.7±2.0	-8.5±1.8	-8.5±1.8	-8.9±1.1
		EL	-6.5±1.9	-10.8±1.1	-9.7±1.4	-10.1±0.7
'Pohjola's Daughter'	15C/14h	visual	-11.6±2.2	-13.5±1.9	-12.5±1.8	-12.3±1.1
		EL	-9.4±1.0	-13.9±1.1	-14.3±3.2	-14.3±3.1
	15C/20h	visual	-7.5±2.5	-8.5±1.8	-9.5±1.8	-8.2±1.1
		EL	-7.0±1.1	-11.9±1.8	-9.7±0.9	-10.4±1.0
	24C/14h	visual	-7.5±2.5	-13.5±1.9	-13.5±1.8	-15.0±1.1
		EL	-14.9±11.0	--[1]	-14.0±1.4	-10.9±1.2
	24C/20h	visual	-6.5±2.1	-11.5±1.9	-10.5±1.8	-10.2±1.2
		EL	-7.4±0.8	-11.9±1.6	-11.3±0.8	-11.4±1.9

[1] Logistic curve fitting unsatisfactory

survival and minor damage were related to reduced elongation growth, but also to low night temperature in early fall (Nilsson 1988). Low night temperature, which, however, was not studied this time, was found to hasten hardening also in *Viburnum plicatum* f. *tomentosum* (Thunb.) Miq. (Irving and Lanphear 1967) and in various conifers (McGuire and Flint 1962), but in *Pinus radiata* D. Don low night temperatures did not enhance hardening (Greer and Warrington 1982).

4.3. Cold hardiness of diploid and autotetraploid rhododendrons (IV, V)

By the end of the summer the diploid and tetraploid rhododendrons, which were originally the same size, had grown to different extents. 'PJM' plants had made two flushes of growth and were approximately 1.3-1.4 times as tall as 'Northern Starburst', which had one flush of growth. The same trend was seen in the ‘Cunningham’s White’ clones. The tetraploid clones had markedly thicker leaves and larger cells than the corresponding diploids.

Before acclimation in the phytotrons the leaves of diploid and tetraploid ‘Cunningham’s White’ plants were equally hardy. Thereafter the diploid clone (CW2) hardened more than the tetraploid one (CW4). The results for visual assessment of injury, EL test and IS were similar, although the levels of hardiness identified by the tests varied (Table 2, Fig. 3 in IV and Fig. 2 in V). The EL test indicated slightly better hardiness and the IS test 2 to 3 degrees weaker hardiness than the visual test. According to the visual test the leaves of ‘Cunningham’s White’ tolerated –35°C and it is uncertain if they had reached maximum hardiness level, whereas the tetraploid CW4 leaves were possibly unable to harden beyond –23°C, since the hardiness did not notably improve at the last acclimation temperature. To my knowledge the leaf hardiness of 'Cunningham's White' has not been previously reported. Although considered to be hardy in Germany, this cultivar is often damaged during winter in Finland.

At the first freezing test date, the LT_{50} value of the diploid 'PJM' from the visual test was –37.7°C and that of its tetraploid derivative 'Northern Starburst' –20.5°C (Table 2, Fig. 3 in IV). 'Northern Starburst' acclimated at +5°C to its final hardiness, –34.2°C. The hardiness of 'PJM' did not change before acclimation at sub-freezing temperature, when it reached –47.1°C. Thus during the acclimation period the two cultivars were partly equally hardy. As determined by IS, 'PJM' was always hardier than 'Northern Starburst'. The hardiness estimates from IS did not change much during the acclimation period. At the end of the experiment, IS derived values indicated considerably weaker hardiness for 'PJM' and 'Northern Starburst' than the visual test. The EL test indicated weaker hardiness than the visual test for these lepidote clones in contrast to the elepidote clones.

Stems of 'PJM' were slightly hardier than those of 'Northern Starburst'. The differences between the cultivars or between visual and EL tests were non-significant, but the difference between the two test dates was significant, the later date indicating weaker hardiness. The stem hardiness varied from –33.3 to –40.1°C (Table 1 in IV). For the stems and leaves of 'PJM', the present results are consistent with those of Sakai et al. (1986), who observed the hardiness of the xylem to be –35 or –40°C and of leaves –30 or –50°C, depending on origin. Roots of container grown 'PJM' have been found to be hardy to –23°C (Havis 1976). ‘Northern Starburst’ is a new cultivar, the hardiness of which has been previously reported only in nursery catalogs, where its hardiness is rated to USDA zone 4 covering minimum temperatures –29 to –34°C. Our results on leaves and stems agree with this rating.

Table 2. Cold hardiness estimates (°C) with 95% confidence intervals of diploid and corresponding autotetraploid rhododendrons assessed visually, by electrolytic leakage test (EL) and impedance spectroscopy (IS).

Test	'Cunningham's White' (CW2)	CW4	'PJM'	'Northern Starburst'
	acclimated at 5-10°C			
visual	-14.0±2.1	-12.8±2.1	-37.7±1.1	-20.5±2.1
EL	-17.5±1.8	-13.6±0.5	-35.0±10.0	--[1]
IS τ	-10.0±0.3	-8.7±1.0	-18.6±4.0	-14.1±3.5
IS r_e	-9.5±1.4	-7.9±0.9	-27.8±7.1	-16.5±3.7
	acclimated at 5°C			
visual	-17.2±0.8	-13.3±0.8	-36.1±1.4	-34.2±0.9
EL	-22.0±0.7	-18.3±0.4	-34.7±6.5	-30.1±3.2
IS τ	-14.3±1.6	-12.0±1.5	-29.0±3.9	-16.6±3.5
IS r_e	-14.0±1.5	-10.6±1.2	-24.6±3.7	-15.6±2.3
	acclimated at 1°C			
visual	-26.0±0.9	-21.3±0.9	-36.1±1.4	-29.8±0.8
EL	--[2]	--[2]	--[2]	--[2]
IS τ	-22.4±2.3	-14.3±2.5	-30.4±4.5	-20.4±1.5
IS r_e	-21.4±2.3	-12.9±2.6	-32.5±5.1	-17.8±3.4
	acclimated at -2°C			
visual	-35.0±1.0	-22.7±0.9	-47.1±0.9	-34.2±0.9
EL	-36.5±1.8	-26.1±1.2	-29.7±4.5	-31.9±2.1
IS τ	-33.6±11.9	-20.8±4.8	-28.6±2.8	-20.3±2.5
IS r_e	-30.4±7.3	-21.0±3.8	-28.8±2.8	-20.9±3.6

[1] Logistic curve fitting unsatisfactory, [2] EL was not measured.

The florets of the tetraploid 'Northern Starburst' were markedly less hardy than those of the diploid 'PJM'. The mean hardiness value for 'PJM' florets was –11.1°C (SE 0.52°C), and the hardiest floret froze at –23.7°C (Fig. 4 in IV). The mean hardiness of 'Northern Starburst' florets was –8.1°C (SE 0.21°C), and the hardiest floret froze already at –14.2°C. The tetraploid florets were slightly larger. Within a genus, smaller primordia or pistil size is often related to better cold hardiness, but does not alone explain it (Sakai and Larcher 1987, Flinn and Ashworth 1999). Large cell size and high water content may explain the reduced cold hardiness of induced polyploids (Levitt 1980, Limin and Fowler 1989).

The florets were apparently unable to show their hardening capacity in these experiments, since according to Salley and Greer (1992) 'PJM' is hardy to –32°C, and Sakai et al. (1986) reported an LT_{40} for its flower buds being –27°C. Either the humidity in the phytotron was too high or the temperature was not low enough. The lowest acclimation temperature –2°C was close to the values (0 to –2°C) effectively inducing not only hardening but also dehardening in azalea flower buds (Pellett et al. 1991). The exotherms of 'PJM' especially were widely distributed, indicating an early stage of acclimation (Kaku et al. 1981, Iwaya-Inoue

and Kaku 1983). However, even hardy buds containing several florets have a broad exotherm span (Graham and Mullin 1976a, Iwaya-Inoue and Kaku 1983).

4.4. Effect of acclimation regime on cold acclimation (III, IV, V)

Most of the 'Pohjola´s Daughter', but only the least hardy 'Helsinki University' plants hardened when moved to +5°C. The plants grown in SD responded better to the decreasing temperature regime than those grown in LD, despite all having been in a 12-hour photoperiod at 9°C for two weeks before the low temperature acclimation regime. For both cultivars, plants that had already hardened to some extent required subzero temperatures for further hardening. The hardiness of the 'Pohjola´s Daughter' plants grown at 15°C did not markedly proceed during the acclimation regime.

The diploid 'Cunningham's White' increased in hardiness in each step of acclimation (Table 2) (IV). The very hardy diploid 'PJM' did not increase in hardiness as long as it was in non-freezing temperatures, but attained a hardiness level of –47 °C when kept at –2 °C for three weeks. The tetraploid CW4 hardened at +1, but exposure to –2 °C barely increased its hardiness. The other tetraploid, 'Northern Starburst', hardened at +5°C, but not further even at subfreezing temperature. Thus the tetraploid clones had possibly reached their maximum hardiness already before transfer to –2°C. When detected by IS the elepidote clones CW2 and CW4 hardened throughout the acclimation regime, whereas the lepidotes 'PJM' and 'Northern Starburst' acclimated only a little. In the experiment described in papers IV and V the plants had been in equal growing conditions, first in natural daylength until the early November and thereafter at a six-hour photoperiod.

Both SD and low temperature can induce cold hardiness in woody plants. SD induces growth cessation and was found to play a greater role than low temperature in the acclimation of various conifers (Aronsson 1975, Bervaes et al. 1978, Christersson 1978, Puttonen and Arnott 1994), whereas low temperature was more effective for apple bark (*Malus sylvestris* Mill.), and xylem of *Alnus* Miller and *Pinus* species (Bervaes et al. 1978, Greer 1983a, Tremblay and Lalonde 1987). Hardiness of *Acer negundo* L. and *Viburnum plicatum* f. *tomentosum* developed linearly with time in SD treatment. The response was more rapid after a preceding SD treatment than after a LD treatment (Irving and Lanphear 1967).

The maximum hardening of woody plants is usually dependent on exposure to freezing temperatures (Weiser 1970). This has been observed for example in *Pinus radiata* (Greer and Warrington 1982) and *Cornus stolonifera* (van Huystee et al. 1967). However, less hardy *Cornus stolonifera* plants responded little to the frost treatment (Harrison et al. 1978). In *Pinus radiata* the hardening rate was linearly regulated by temperature (Greer 1983a), but Zehnder and Lanphear (1966) did not find any increase in hardiness of *Taxus cuspidata* Sieb. & Zucc. with falling night temperatures nor any change below a certain temperature level or duration.

The hardening regimes in paper III and on the other hand in IV and V had the same temperatures, but differed in duration. In paper III each step lasted for two weeks, in IV and V for three weeks. In addition, in the earlier experiment the daylength declined abruptly from 20 to 12 h, whereas in the latter experiment the plants were allowed to start acclimating gradually under natural daylength until the beginning of November. Naturally hardened

Taxus cuspidata plants developed better hardiness than artificially hardened ones (Zehnder and Lanphear 1966).

4.5. Plant age and hardiness

The phytotron studies were mostly done with juvenile one- or two-year-old plants. In addition to the mature field grown plants in paper I, only the azaleas studied for growth parameters in paper II were older. Age could partly explain why they ceased growth after one flush, except for *R. canadense* at an unnaturally high temperature, while most of the younger evergreen rhododendrons produced several flushes. For many woody species the seasonal period of elongation growth is shorter in mature plants than in seedlings (Wareing 1956, Pettersen 1972, Håbjørg 1978, Junttila and Kaurin 1985, Ununger et al. 1988). After transition from juvenile to mature growth rhythm other factors may override the photoperiodic effects, e.g. competition between various shoots and preparation for flowering (Wareing 1956).

At best the leaves of 'Helsinki University' became hardy only to –19.7 and 'Pohjola's Daughter' to –15.0°C. These values are far from those the plants can tolerate in the field. On the other hand, equally young 'Cunningham's White' leaves tolerated –35°C and its tetraploid derivative –23°C. Since 'Cunningham's White' flower buds are said to be hardy down to –26°C (Salley and Greer 1992), the young plants may have attained the hardiness of mature plants. For the two-year-old 'PJM' leaves and stems our results are in agreement with those of Sakai et al. (1986), who found the hardiness of the xylem of a mature plant to be –40°C and that of leaves –50°C. From another source in the same study, the values for xylem and leaves were –35 and –30°C, respectively, but the age of the plants was not mentioned.

Sometimes hardiness surveys of juvenile *Rhododendron* plants underestimate the real cold tolerance of the mature plants (Lim et al. 1998a, 1999). Mature *R. maximum* plants were found to be approximately 9°C hardier than three-year-old seedlings, and same trend was seen in cultivars 'Hawaii', 'Swansdown' and 'Pink Parasol' (Lim et al. 1999). However, the effect can not be generalized and juvenile plants may be as hardy as older ones (Sakai and Larcher 1987).

4.6. Comparison of methods of evaluating injury

Ever since Dexter et al. (1932) introduced the electrolyte leakage test it has been widely used in quantifying injury to plant tissues after a freeze-thaw stress. Usually two or more different tests are used contemporaneously, and quantitative results are compared with regrowth or visual assessment of injury. In this study EL was compared with visual evaluation in detecting injury in stems (I, IV) and leaves (III, IV). In addition, IS was compared with visual assessment of leaves (IV, V). The results on the leaves are compiled in Tables 1 and 2.

Cold hardiness estimates of azalea stems (I) provided by the two tests were identical in half of the cases and varied little in the other half, but neither test gave consistently better estimates. Furthermore, stem hardiness of 'PJM' and 'Northern Starburst' was similar in the two tests (IV). Sometimes stem tissues may be heterogeneous to such an extent that EL tests are unusable, as ions leaking from various tissues mask those from the vital tissues, such as living ray parenchyma. Stems of commonly grown elepidote rhododendrons are characterized by thick bark, but the twigs of deciduous azaleas and lepidote rhododendrons, that were

studied, consist principally of xylem and only a thin bark. They seemed to be suitable for EL assessment. For the stems of 'PJM' and 'Northern Starburst' the EL test gave more accurate cold hardiness estimates than the visual test: the widths of the 95% confidence intervals were 1.2 to 1.6°C in the EL test and 3.0 to 3.6°C in the visual test.

The results from visual and EL tests gave slightly different hardiness estimates for leaves. Usually the EL test indicated better hardiness than the visual assessment for the elepidote cultivars 'Helsinki University', 'Pohjola´s Daughter' and both 'Cunningham's White' clones, but the reverse was noted for 'PJM' and 'Northern Starburst'. In other studies with evergreen leaves milder injury has been detected visually than by EL measurements (Gu et al. 1987, Lindstrom 1992).

Two cold hardiness estimates were calculated from the impedance analysis, one based on extracellular resistance and the other on relaxation time. They correlated well with each other and with the results of visual scoring, but indicated generally weaker hardiness than the visual test. This was expected, since IS can detect minor changes in the cellular structure. The same relationship has been found in willow (*Salix viminalis* L.) stems (Repo et al. 1997). The difference between IS and visual tests was smaller and more coherent within the 'Cunningham's White' clones than within 'PJM' and 'Northern Starburst'. The latter ones accumulated dark pigments in their leaves, which generated difficulties and possibly bias also in the visual rating of damage.

In this work the LT_{50} estimates of cold hardiness are presented with their 95% confidence intervals, as also used by Burr et al. (1990). Most often cold hardiness is presented as a single estimate LT_{50}. However, the transition from no injury to complete kill has been found to occur within a range of 1-6°C in twigs of fruit trees (Ketchie et al. 1972) and 1-4°C in evergreen leaves (Neuner and Bannister 1995).

The accuracy that can be obtained for cold hardiness estimates varies between species, types of tissue, acclimation intensity and methods of analysis. The width of the 95% confidence interval in IS tests was on average 6.5°C. 'PJM' had the widest and CW4 the narrowest span. In EL tests of the leaves the span was on average 2.1°C for 'Cunningham's White' clones, 5.6°C for 'Helsinki University' and 'Pohjola's Daughter', and 10.6°C for 'PJM' and 'Northern Starburst'. In the visual assessment of the leaves the 95% confidence intervals for the least hardy plants extended over 4 to 5°C in both experiments, partly due to variations among leaves at an early hardening stage. The confidence intervals became narrower when hardening proceeded, and extended over 1.7 to 2°C at the end of the experiments. At the first test date 'PJM' had a narrower confidence interval than the other clones, but at the later stages of acclimation there were no clear differences between clones. No clear trend was seen in either EL or IS test.

The more accurate cold hardiness estimates in the visual test than in EL and IS tests were due to different statistical analyses applied to quantitative (for example from EL or IS analysis) and binomial data. In EL and IS tests the sigmoid function was fitted to the data separately for each clone and freezing test date combination. The relatively small number of data points resulted in large standard errors and, consequently, wide confidence intervals. The sigmoid response curve is usually steep for non-acclimated tissues, and flattens with hardening. The declining slope over time indicates a decrease in sensitivity to changing temperature with increased cold hardiness. In other words, any decline in temperature causes less injury to acclimated than to non-acclimated tissue. Hardiness estimates are most precise when the

slope is steep and cold hardiness is minimal (Burr et al. 1990). Sometimes wide confidence limits are caused by inaccurate estimation of the asymptotes due to an insufficient temperature range or sample size, or scattering of the data at each freeze test temperature indicating differences among samples.

In logit analyses, all data were combined, which resulted in more accurate estimates. Furthermore, the visually detected threshold level of damage (50%) is considered lethal based on my own and others' empirical experience (Holt and Pellett 1981, Johnson and Hirsh 1995). Serious damage may also be seen as browning of the midrib and veins. This score also correlated better with the EL tests than 10 or 90% damage (III). In outdoor plantings damage to leaves of evergreen plants is seen as brown veins, leaf-edges or whole leaves and later abscission reducing the ornamental value of the plant. According to Johnson and Hirsh (1995) the hardiness of evergreen species is best defined as evergreeness, when plants are rated evergreen if less than 50% of the leaves are discolored. The inflection point of the sigmoid curve of quantitative data is commonly regarded as the killing point without knowing which part of the tissue is damaged. However, both binomial and quantitative data are useful, since researchers are usually more interested in the relative rather than absolute hardiness of plants.

Florets of *Rhododendron* are easy to score visually as dead or alive, and since they die at the moment they freeze, their killing point can be accurately detected with DTA or NMR. In paper I, the hardiness of florets was given as the lowest survival temperature (LST) (Sakai et al. 1986), where the threshold between alive and dead was 50%. The hardiness values obtained may underestimate hardiness by less than two degrees, except for the first test date in mid-November, when the samples were taken out from the freezer at three-degree intervals rather than the later two-degree intervals. In paper V, flower bud hardiness was detected using DTA giving an exact temperature value for the killing point of each floret. These data were analyzed with ANOVA, and confidence of the mean hardiness estimate was obtained as a standard error. In 'PJM' mean hardiness was 1.1°C lower than the LT_{50} value, whereas in 'Northern Starburst' the difference was negligible.

4.7. Predicting hardiness without freezing tests (V)

In unfrozen rhododendron leaves two IS parameters, extracellular and intracellular resistance, r_e and r_i respectively, increased during hardening. The changes were more clearly seen in the elepidote 'Cunningham's White' clones, than in 'PJM' and 'Northern Starburst' (Fig. 3 in V). Thus they could be used in hardiness determination of the elepidote clones, where they correlated with IS derived cold hardiness estimates (Table 1 in V). However, testing without freezing does not provide numerical hardiness estimates, but only information about changes in tissue properties, since the parameter values of hardened tissues are compared with the values of non-acclimated tissues. Although the correlation in lepidote clones was non-significant, it seems that the method may be improved to suit them also. In Scots pine (Repo et al. 1995) and willow stems (Repo et al. 1997) r_i followed the changes in hardiness. In willows, alterations in IS parameters were suggested to be connected with cell differentiation and lignification of wood, as well as dry mass content and unsaturated:saturated ratio of fatty acids (Repo et al. 1997). In *Pinus radiata* seedlings the relationship between electrical impedance of unfrozen stems and frost hardiness was clear as long as the plants were in an actively hardening stage, but impedance continued to increase even though maximum hardiness was attained (Greer 1983b). The relationship was less clear during dehardening.

5. CONCLUSIONS

In northern Europe cold winters, fluctuating temperatures, unstable snow cover, cool summers and short growing seasons with late and early frosts restrict the growing of *Rhododendron*. In addition, the long daylength in summer should be regarded as a restricting element. Attention should be paid to proper timing of cold acclimation when marketing *Rhododendron* across the Atlantic in spite of the fact that the winters can be equally cold on both sides of the ocean. Due to different latitudes the light climates of the corresponding growing zones in these two continents differ considerably. In addition, summer temperatures are usually higher and the growing season is longer in America than in northern Europe.

This study showed that cold hardiness of *Rhododendron* is partly dependent on the growing season characteristics. Both LD and high temperature interfered with the growth, and triggered the plants to continue growing beyond the typical one flush per summer. However, genotypes reacted differently to applied daylength and temperature treatments. Of the two cultivars tested 'Helsinki University' benefited from a cool summer typical for Scandinavia, whereas the photoperiod played a greater role than temperature in the cold acclimation of 'Pohjola´s Daughter'. 'Helsinki University' plants were hardier than 'Pohjola´s Daughter' when plants were grown at 15°C, but after the summer at 24°C the 'Pohjola´s Daughter' plants proved superior.

In breeding *Rhododendron*, attention should be paid to growth rhythm and timing of cold acclimation at various latitudes. Day-neutral plants that make only one flush in moderate temperature regardless of photoperiod would be best for northern areas. The cultivars that are very hardy in northern areas should be tested also in milder or more southern locations. A plant that is hardy in certain cold areas may get severe injuries in other areas even with milder winters, especially if the growth continues for a long time in late summer or fall. For only moderately hardy deciduous azaleas or other *Rhododendron* breeding for better midwinter hardiness of flower buds can be effective and profitable. Flower buds of deciduous azaleas acclimated later and deacclimated earlier than the stems of the same plants.

Several ornamental characters of *Rhododendron* can be improved through polyploidy, but unfortunately polyploidization did not prove to be a useful breeding method for increasing, or maintaining cold hardiness. The leaves and florets of the induced tetraploids were less hardy than the diploids they were derived from, but no difference was found between the stems of the corresponding clones. However, even though the hardiness decreased, the timing of acclimation was not affected by doubling the chromosome number. Thus, the method could be used to enhance new ornamental characteristics in milder areas.

The present results indicate that rhododendron leaves can be scored as dead or alive on the basis of 50% browning or severe damage to the midrib and major veins. Furthermore, more accurate hardiness estimates were obtained using visual assessment of injury than using the quantitative methods EL and IS. The visual method benefited from the statistical analysis applied, since all data could be combined in logit analysis rather than analyzing each cultivar separately from each freezing test. IS was an adequate method for studying frost hardiness of elepidote rhododendrons both after controlled freeze tests and without artificial freezing. However, the results from the lepidote clones were less consistent with those from visual assessment and the parameters of the unfrozen samples did not correlate with frost hardiness. Further research is needed to obtain better fit and more satisfactory interpretation of the lepidote data.

ACKNOWLEDGEMENTS

This study was carried out at the Department of Plant Biology of the University of Helsinki, although the various experiments were conducted at other institutes: the Horticultural Research Center of the University of Minnesota, the Department of Biology of the University of Tromsø and the Faculty of Forestry of the University of Joensuu. I thank all these institutes for providing their facilities, and the staff for their kind assistance.

I want to thank several people who have contributed to this work in one way or another. First of all, I am grateful to my two supervisors. Professor emeritus Peter Tigerstedt from the University of Helsinki introduced me to the genus *Rhododendron* back in 1986 and has ever since encouraged me in breeding and research work. My sincere thanks are due to him also for contributing in organizing the financing. Professor Olavi Junttila from the University of Tromsø has generously offered his help in guiding and advising me during the course of the study. Dr. Marja-Liisa Sutinen and Dr. Elina Vapaavuori are acknowledged for their careful work in reviewing the manuscript and Dr. Jonathan Robinson for revising the English.

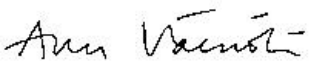
My warmest thanks are also due to Dr. Tapani Repo for rewarding collaboration and co-authoring two papers. As well, the American co-authors Dr. Harold Pellett and M. Sc. Steve McNamara are acknowledged for fruitful collaboration. Thanks are due to M. Sc. Matti Haapanen and Dr. Hannu Rita for their help in statistical analyses.

I thank my colleagues at the Department of Plant Biology for sharing every day life. Especially, I want to thank Terttu Parkkari for friendship, encouragement and together with Marjo Toivonen for technical assistance. Colleagues in the unique 'Cold Group', Leena Lindén, Pauliina Palonen and Mervi Seppänen, have afforded me great support by offering a field for all kinds of discussions over these years.

Finally, I would like to thank my friends and family members for their interest and understanding attitude to my work.

The study was supported by the Department of Plant Biology of the University of Helsinki, the Association of Academic Agronomists, the Emil Aaltonen Foundation, Konkordialiitto, the Nordic Academy for Advanced Study (NorFA) and the Rector of the University of Helsinki. Plants were received from Puutarha Tahvoset and Briggs Nursery, Inc. These are all gratefully acknowledged.

Helsinki, April 2000



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