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**Physiological study of cold acclimation
in *Rhododendron sp.* with emphasis on role of dehydrins**

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**Thesis submitted to the College of
Agriculture, Forestry and Consumer Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Master of Science
in
Plant and Soil Sciences**

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**Morgantown, West Virginia
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**Keywords: Abscisic Acid, Cold Acclimation, Dehydrin, Photoperiod, *Rhododendron*, Water
Stress, Woody Plants**

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ABSTRACT

Physiological study of cold acclimation in *Rhododendron* sp. with emphasis on role of dehydrins

Calin O. Marian

In this study we established the significance of 25 kDa dehydrin accumulation during cold acclimation (CA) in a wide array of *Rhododendron* species. These species (24 in total) belong to two diverse subgenera, *Hymenanthus* and *Rhododendron*, native to diverse latitudes and altitudes. The dehydrin of interest is highly conserved in *Rhododendron* genus and was present and up-regulated during CA in all the species studied with one exception - *R. brookeanum* - a species adapted to tropics. Some other dehydrins were also found to accumulate in response to cold acclimation in several species, but none of these accumulated consistently. Experimental data show that there is no correlation between the absolute amount of 25 kDa dehydrin and the degree of leaf hardiness in cold acclimated plants. Moreover, a higher number of dehydrin species in a particular genotype does not necessarily translate into more hardy *Rhododendron*. However, our results suggest that the cold-inducibility of a 25 kDa dehydrin is positively correlated with cold acclimation ability in *Rhododendron*. The 25 kDa dehydrin appeared not to be specifically regulated by cold - its accumulation was triggered by water stress as well. However, we were unable to detect any accumulation of this dehydrin in response to exogenous abscisic acid (ABA) application. During the first stage of cold acclimation, the accumulation of 25 kDa dehydrin is triggered by short photoperiods. However, the onset of cold temperatures during late Fall and winter overrides the early photoperiod stimulus. Our data also showed that short photoperiod alone (in the absence of low temperature) is sufficient to induce both a small level of cold acclimation and 25 kDa accumulation in *Rhododendron* leaves. However, plants are unable to cold acclimate to any relevant level when exposed to warm temperatures and extended day-length irrespective of exposure to seasonal shifts over the year. Tissue localization studies indicate that 25 kDa dehydrin accumulates in the leaf lamina with lower accumulation in the midrib. This may be correlated with the primary area of injury in *Rhododendron* as being the midrib.

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CHAPTER ONE: INTRODUCTION

The ability of various *Rhododendron* cultivars and species to tolerate winter temperatures is probably the most limiting factor for their use in many areas of the world. In most agricultural areas, unseasonal frost can occur throughout much of the growing season. Depending on the temperature and the duration of the frost, plants may be partially damaged or killed. This accounts for sizeable losses (10-50% reduction in sales) to ornamental plant industry in West Virginia (WVU Extension Service). At national level, the economic losses due to freezing injury to crop plants and ornamental plants including woody ornamentals exceed \$ 1 billion annually (White and Hass 1975).

Perennial plants growing in temperate zones have the ability to develop increased cold hardiness when exposed to hardening conditions (during autumn, reaching a maximum in the winter). This process, known as cold acclimation is considered the most important factor for mid-winter survival. The opposite process (decrease of tolerance to low temperature during spring, reaching a minimum in the summer), called deacclimation, is equally important. Untimely mid-winter hard freeze and/or late spring frosts followed by thawing can cause severe damage to the flower buds and/or ornamental foliage of many horticultural important crops, including *Rhododendron*.

Understanding the processes of cold acclimation and deacclimation in broadleaf evergreens such as *Rhododendron* is very important for advancing our understanding of the physiological basis of these two events. Ultimately, this may lead to the development of new, cold hardy germplasm and improved cultural practices, which may induce greater mid-winter and spring hardiness.

LITERATURE REVIEW

Freezing injury and cold acclimation

In nature, plants cool slowly, usually only a few degrees per hour in the most extreme situations, and thaw at an equally slow rate (Levitt 1980). During extracellular freezing, ice forms between cells, which in turn initiates the removal of free water from cells in order to come to vapor pressure equilibrium. This leads to dehydrative stress in the cells (Sakai and Larcher 1987; Uemura and Steponkus 1999). Freezing injury is regarded to be a consequence of membrane lesions that are caused by the dehydration that occurs during freezing (Steponkus 1984), although other factors may also contribute to the cellular damage induced by freezing (Thomashow 1999). In contrast to extracellular freezing, intracellular ice formation that occurs at fast cooling rates is lethal to the cell because the formation of ice crystals leads to mechanical disruption of cell structures.

Perennial plants growing in the temperate regions undergo a "hardening" process in the fall of each year to prepare for overwintering. Cold acclimation is the term used to describe the transition from tender to hardy status. There are a number of morphological, biochemical and biophysical changes associated with the induction of freezing tolerance during cold acclimation. The cold acclimation process occurs in at least two phases. The first phase is induced by short day photoperiod (Fuchigami et al. 1971; Gray 1997) while the second phase is induced by increasingly low temperatures. In some hardy woody species, a third phase may occur, requiring sub-freezing temperatures in order to achieve full cold hardiness potential (Weiser 1970).

Usefulness of *Rhododendron* for cold acclimation studies

Evergreen plants need to be able to fully utilize the growing season, yet commence hardening sufficiently early to withstand fall frosts, and reach a sufficiently deep winter hardiness to withstand extreme cold events. Because winter dormancy and cold acclimation are

overlapping events in most woody perennials, the physiological and molecular processes involved in them are difficult to distinguish and separate. In evergreen plants, however, cold acclimation can occur in tissues (leaves) that are not endo-dormant, allowing a separation to be made between these two events. This feature makes *Rhododendron*, a broad leaf evergreen, suitable as an experimental system to study cold acclimation processes. Physiological understanding of CA is not only of great interest scientifically, but also has economic implications because it may allow manipulation of plant cold hardiness and reduce frost damage.

Moreover, within the *Rhododendron* genus, the ability to withstand severe low temperatures varies widely. Sakai et al. (1986) found that many species in the *Ponticum* subsection (*R. brachycarpum* and *R. maximum*) are leaf-hardy to -60°C and bud-hardy to -30°C , whereas cold-tender species, such as *R. barbatum* and *R. griersonianum*, show both leaf and bud damage at temperatures approaching -18°C . The ability of *Rhododendron* to withstand such low temperatures makes this species an ideal material to study the freezing tolerance and acclimation to extremely low temperatures.

Environmental factors and cold acclimation in *Rhododendron*

The effect of photoperiod on the onset of dormancy and initiation of cold acclimation is well documented in the literature. In trees and other woody perennial plants, short days induce growth cessation, initiation of cold acclimation and bud dormancy (Teets et al. 1989; Fennell and Hoover 1991; Lu and Rieger 1990; Howe et al. 1995; Welling et al. 1997). In evergreen woody plants, such as *Rhododendron*, short photoperiod appears to be directly related to the initiation of cold hardiness process (Vainola et al. 1999; Cameron and Dixon 2000). Plants grown under short photoperiod developed an increased cold hardiness but there is a strong interaction between short days and low temperatures. When these two factors were combined, the plants achieved maximum cold hardiness. While short days appear to be important in the first stage of cold acclimation, low temperatures will eventually override the light stimulus (Cameron and Dixon 2000).

Dehydrins

An important group of proteins that typically accumulate in plants in response to dehydration was first detected in maturing seeds and categorized as Late Embryogenesis Abundant (LEA) proteins. These proteins are divided into groups based on specific amino-acid sequences (Bray 1993; Dure 1993). The D-11 family (Dure 1993) or group 2 LEA proteins (Bray 1993) are commonly known as dehydrins. These proteins are induced in plants by environmental stresses that have a dehydrative component such as low temperature, drought, high salinity and even wounding (Close 1996; Richard et al. 2000).

Dehydrins are characterized by the presence of one or several lysine-rich units called the K-segments conserved near the carboxy terminus and repeated several times throughout the sequence (Close 1996). Another consensus sequence (DEYGNP), the Y-segment, can be found near the amino terminus of most of the dehydrins (Close 1996). The 15-amino-acid consensus sequence of the Lys-rich motif EKKGIMDKIKEKLPG has been used to produce specific antibodies that recognize dehydrins in a wide range of plants (Close et al. 1993). Several proteins from different species, including woody perennials, have been detected with this anti-dehydrin antibody (Close et al. 1993; Wisniewski et al. 1996; Lim et al. 1999).

Accumulation of dehydrin proteins and transcripts during cold acclimation has been amply documented in a number of herbaceous species (Guy et al. 1994; Close 1997; Thomashow et al. 1998), but its association with cold acclimation in woody perennials, which exhibit significantly higher acclimation ability and freezing tolerance than herbaceous plants, are comparatively scarce. Studies of deciduous woody species documented dehydrin profiles in overwintering tissues such as xylem, bark, and floral buds (Arora and Wisniewski 1994; Muthalif and Rowland 1994; Salzman et al. 1996; Artlip et al. 1997; Welling et al. 1997). In deciduous peach trees, freeze-hardiness of bark tissues was positively correlated with the accumulation of a 60 kDa dehydrin protein (Arora and Wisniewski 1994). In evergreen woody species, dehydrin proteins and genes have been identified in conifer species such as *Pinus* (Close et al. 1993) and *Pseudotsuga* (Jarvis et al. 1996) as well as in evergreen peach (Arora and Wisniewski 1994). Several dehydrins were detected in *Rhododendron* leaves and a 25 kDa dehydrin appears to be positively associated with freezing tolerance and believed to serve as a genetic marker for cold hardiness (Lim et al. 1999).

Dehydrins are found to accumulate differentially in plant tissues. For example, some of these proteins have been found to preferentially accumulate in the provascular and vascular tissues of tomato (Godoy et al. 1994), in xylem tissues of peach (Arora and Wisniewski 1996), vascular bundles and epidermis of barley (Bravo et al. 1999). The association of dehydrins with vascular tissues could be related to the evidence that ice crystal formation occurs in the vascular bundles, causing water migration from the nearest cells to compensate for the water vapor deficit of ice. At subcellular levels, dehydrins are localized in the cytoplasm or nucleus (Close 1996) but recently dehydrin-like proteins have been found in storage protein bodies and amyloplasts of cold acclimating *Betula pubescens* (Rinne et al. 1999), plastids of *Prunus persica* bark (Wisniewski et al. 1999) and associated with plasma membrane in wheat (Sarhan 1997). Cold acclimation also induces their accumulation in the mitochondria of several plant species (Borovskii et al. 2000).

Several attempts were made in order to elucidate the role of dehydrins in plants. From a physiological perspective, the tolerance of protoplasm to desiccation is an important aspect of plant survival because it leads to an increased capacity of plants to withstand drought and winter cold. The low freezing temperature is perceived at cellular level as a dehydrative stress, because the freezing of extracellular water forces the water out of the cell due to strong vapor pressure gradient. Therefore, during cold acclimation process, dehydrins are expected to accumulate in the plant cells.

A functional role of dehydrins is suggested by their hydrophilic nature (thereby protecting macromolecular structures from desiccation) and *in vitro* cryoprotectant properties (Lin and Thomashow 1992; Close 1996; Ingram and Bartels 1996; Wisniewski et al. 1999). The peach (*Prunus persica*) PCA60 dehydrin possesses antifreeze activity thereby altering the shape of the ice crystals, which could aid in reducing the freezing damage to the cells (Wisniewski et al. 1999). Cryoprotective activity of this dehydrin on lactate dehydrogenase enzyme function has also been demonstrated (Wisniewski et al. 1999). Rinne et al. (1999) found that under low water conditions, RAB-16-like dehydrins from birch (*Betula pubescens*) were able to improve the activity of α -amylase. Other possible functions of dehydrins include ion-sequesters (Palva and Heino 1998) or molecular chaperones (Campbell and Close 1997; Close 1997) under stressful conditions, thereby stabilizing proteins and cell membranes.

Several studies established a positive correlation between dehydrin accumulation and cold hardiness among genotypes (Danyluk et al. 1994; Muthalif and Rowland 1994; Robertson et al. 1994; Cai et al. 1995; Arora et al. 1997; Artlip et al. 1997). By over-expressing a constitutive gene that regulates the expression of COR (cold regulated) proteins, some of which are dehydrins, Jaglo-Ottosen et al. (1998) showed that COR genes promote the freezing tolerance in *Arabidopsis*.

Previous studies in our laboratory with *Rhododendron* populations segregating for cold hardiness indicated that leaf freeze tolerance increases with both chronological age and developmental phase-change and this trend is accompanied by increased accumulation of a 25 kDa dehydrin (Lim et al. 1999). It was further suggested that the presence or absence of the 25 kDa dehydrin could serve as a genetic marker to distinguish between super hardy and less hardy *Rhododendron* genotypes. The 25 kDa dehydrin that is accumulating in cold acclimated plants of super-hardy species, such as *R. catawbiense*, may have cryoprotectant/ chaperone/ ion-sequestration properties which exceed the higher molecular weight dehydrins found in moderately hardy species such as *R. fortunei* (Lim et al. 1999).

Considering the information provided by previous research in our laboratory, a logical next step is to undertake a broader study and determine if 25kDa dehydrin is associated with cold acclimation in a wide array of species within the genus *Rhododendron*, and whether the quantitative accumulation of this dehydrin correlates with the degree of cold hardiness in these species. This survey may also identify dehydrins that specifically accumulate during cold acclimation in only those *Rhododendron* species that may be genetically and/or evolutionary related. Moreover, to better understand the physiology of cold acclimation in *Rhododendron*, it is important to study the environmental factors (photoperiod, low temperatures, water stress) responsible for dehydrin accumulation and cold acclimation.

RESEARCH OBJECTIVES

There are many unanswered questions about the cold acclimation process in evergreen woody perennials. This study was undertaken to further understand the mechanisms of cold acclimation process in *Rhododendron* in connection with dehydrins accumulation. This species is ideal for this type of study due to its evergreen character and ability to withstand severe cold temperatures. Moreover, there are more than 850 species of *Rhododendron* that provide a large pool of diverse genotypes with varying leaf hardiness. Following are the main objectives of this study. **First**, to survey a wide array of *Rhododendron* species for dehydrin accumulation patterns in cold acclimated plants vs. non-acclimated plants with special emphasis on 25 kDa dehydrin. Previous studies indicated that a 25 kDa dehydrin was useful as a genetic marker for cold hardiness in few hybrid cultivars and segregating F₂ populations. The proposed study would establish (or refute) a more universal role of 25 kDa dehydrin in cold acclimation of *Rhododendron*. Moreover, some other dehydrins might be identified as associated with cold acclimation. **Second**, to establish if the accumulation of 25 kDa is specifically regulated by cold or also by other environmental factors. Therefore, the effect of several environmental factors, such as light, temperature, water stress and ABA application must be assessed. It would also be important to establish in what type of tissues dehydrins accumulates within the plant. These results might provide insights into the protective role of 25 kDa dehydrin in specific plant tissues.

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ORGANIZATION OF THE REMAINING CHAPTERS

The rest of this thesis is divided into three chapters. Chapters two and three are full-length articles that will be submitted for publication in a peer-reviewed journal. Chapter four is a conclusion chapter. Lastly, in the appendix, a brief chapter on deacclimation study of *Rhododendron* can be found. This was initially included in the original plan of study but could not be concluded in its entirety due to time constraints.

CHAPTER TWO: DEHYDRIN SURVEY IN A WIDE ARRAY OF *RHODODENDRON* SPECIES AND THEIR ASSOCIATION WITH COLD ACCLIMATION

Abstract

Dehydrins are hydrophilic and heat-stable proteins that accumulate in plant cells during cold acclimation and are believed to play an important role in stabilizing cellular membranes and macromolecules during freeze-induced dehydrative stress. Previous work on *Rhododendron* indicated that a 25 kDa dehydrin accumulation levels were closely associated with differences in leaf freezing tolerance among F₂ segregants generated from a cross between a super-hardy and moderate hardy *Rhododendron* species. Present investigation focused on studying the presence and accumulation patterns of this dehydrin and surveying other dehydrins in a wide array of *Rhododendron* species (24 in total) belonging to several subgenera, sections, subsections and geo-climatic zones. Experiments were also conducted to study whether accumulation levels of 25 kDa dehydrin are associated with cold acclimation ability in *Rhododendron*. A total of 11 dehydrins were detected across all 24 species and they were all cold-induced. Of these 11 dehydrins, the 25 kDa was the most conserved dehydrin across species. It was present in all *Rhododendron* species except one, which is of tropical origin and lacks cold acclimation ability. The 25 kDa dehydrin accumulated to relatively high levels (compared to non-acclimated plants) during cold acclimation across all species and the quantitative accumulation appears to be linked to cold acclimation ability. Furthermore, experiments with five other genera belonging to *Ericaceae* family revealed that the 25 kDa dehydrin was present and accumulated during cold acclimation only in *Kalmia latifolia*, a species most closely related (phylogenetically) to *Rhododendron*, whereas it was not detected in *Arctostaphylos uva-ursi*, *Vaccinium macrocarpum*, *Leucothoe fontanesiana* and *Pieris floribunda*.

Key words: cold acclimation, dehydrins, *Ericaceae*, *Rhododendron*, woody perennials

Introduction

It is well known that the cellular levels of several organic metabolites increase when plants are exposed to stresses such as water stress, cold stress or salt stress. One of the organic compounds that accumulate in response to stress, with dehydration as a common factor, is a class of proteins called dehydrins. Dehydrins belong to group D11 of a multigene family of proteins called LEA, named after their initial observation as "Late Embryogenesis Abundant" during cotton embryo development (Dure et al. 1989). LEA D-11 family of proteins (or dehydrins) are characterized by a consensus 15 amino acid sequence EKKGIMDKIKEKLPG that is present near the carboxy terminus and in additional copies upstream of the terminus, in many cases, as a slightly modified 14 amino acid consensus: KKGIKEKIKEKLPG (Close 1996).

Dehydrin proteins or transcripts have been shown to accumulate during cold acclimation (CA) in a number of woody plant tissues: peach bark (Arora and Wisniewski 1994; Artlip et al. 1997), peach xylem (Arora and Wisniewski 1996), blueberry buds (Levi et al. 1999; Muthalif et al. 1994), grape buds (Salzman et al. 1994), birch apices (Rinne et al. 1998, 1999). However, in contrast with herbaceous plant species and model systems such as *Arabidopsis*, where some progress has been made in establishing a causal relationship between accumulation of dehydrins or dehydrin-like proteins and freezing tolerance (Jaglo-Ottosen et al. 1998; Artus et al. 1996), their direct role in woody plant hardiness has been less explored and remains elusive.

This has been, in part, due to complex biology of winter survival in woody perennials where seasonal cold acclimation and dormancy events are superimposed on each other. Although attempts have been made to associate dehydrin metabolism specifically with cold acclimation or dormancy by using various systems and strategies (Arora et al. 1997; Salzman, 1994), no causal relationship could be established between dehydrin accumulation and CA. Use of broadleaf evergreens, such as *Rhododendron* offers an opportunity whereby cold acclimation physiology can be studied in a system (such as over-wintering leaf tissues) without interference of endo-dormancy transitions that may occur in other tissues of deciduous woody perennials.

An understanding of the physiological mechanisms of cold acclimation in *Rhododendron* is of great interest to breeders as well as nursery industry because low winter temperature limits the geographic distribution of this species. Severe winter temperatures can cause injury to the

Rhododendron foliage and buds, leading to sizeable economic losses to ornamental plant industry. Understanding these mechanisms may lead to the development of new, cold hardy varieties and improved cultural practices which may induce greater mid-winter hardiness and reduce cold injury during untimely spring frosts.

Lim et al. 1999 first studied the role of dehydrins in *Rhododendron* cold hardiness, using a super-hardy species (*R. catawbiense*), a less hardy species (*R. fortunei*) and their F₂ progenies. They reported that in cold acclimated F₂ progenies, levels of a 25 kDa dehydrin were closely associated with differences in leaf freezing tolerance (LFT) among segregants. They also suggested that the presence or absence of 25 kDa dehydrin could serve as a genetic marker to distinguish between super cold hardy and less cold hardy *Rhododendron*. However, the role of this dehydrin *vis-a-vis* cold acclimation in a wide array of *Rhododendron* species was not addressed in this study. It was also not investigated whether the quantitative accumulation of this dehydrin was associated with varying degrees of cold acclimation ability in *Rhododendron* species.

The research presented here was conducted to study the accumulation pattern of 25 kDa dehydrin during CA in 24 species of *Rhododendron* belonging to different subgenera, sections and subsections native to diverse geo-climatic zones worldwide. Experiments were also conducted to study the association of this dehydrin with the cold acclimation ability in several *Rhododendron* species. Furthermore, we investigated the presence or absence and the association of 25 kDa dehydrin with CA in five other genera of *Ericaceae* family to which *Rhododendron* belongs.

Materials and methods

Plant material

Leaves from several *Rhododendron* species and varieties were obtained from field and container plants maintained at Holden Arboretum's David G. Leach Research Station in Madison, Ohio (Table 1). Cold acclimated leaves were collected during late December and early January whereas the non-acclimated leaves were collected from the same individuals during the summer (July-August). Cold- and non-acclimated leaves from three species of *Ericaceae* (*Kalmia latifolia*, *Leucothoe fontanesiana* and *Pieris floribunda*) were also obtained from field grown plants at the same location as above. Leaf samples for the other two species of the same family (*Arctostaphylos uva-ursi* and *Vaccinium macrocarpum*) were collected in the wild, near Morgantown, West Virginia.

Relative cold hardiness estimation

Leaf freezing tolerance (LFT) was determined through a controlled-freezing protocol in the laboratory and injury was assessed by ion-leakage from leaf tissues. Cooling rates, ion leakage calculations, % injury estimations, Gompertz functions fitting, determination of T_{max} (temperature causing maximum rate of injury and defined as leaf freezing tolerance) and statistical analysis were performed as described by Lim et al. 1998a.

Total protein extraction and estimation

Protein extraction was performed according to Lim et al. 1999 with few modifications. The protein pellets obtained after trichloroacetic acid precipitation of the crude extract were washed three times with cold acetone. After each acetone wash a sterile sealed pipette tip was used to physically break the pellet. Dried protein pellets were re-hydrated with 100 μ l of 0.5x sample solubilization buffer (Owl Separation Systems, 0.125 M Tris-HCl pH 6.8, 1% sodium dodecyl sulfate, 5% mercaptoethanol, 15% glycerol, 0.005 % Bromphenol Blue) followed by

boiling in a water bath for 5 minutes. The Esen method (1978) for determining total protein dissolved in the sample buffer was used, as described by Lim et al. 1999.

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblots were performed according to Lim et al. 1999. In order to establish the optimal amount of protein loading for immunoblots, a saturation curve was constructed by loading 1, 3, 5, 7, 9, 11, 13, 15, 17 μg total protein extracted from *R. catawbiense*. These amounts were plotted against the optical density values of the 25 kDa band detected in the immunoblots and a logarithmic curve was fitted to the data (Fig.1). For all the subsequent immunoblots in this study, 7 μg protein were loaded on the gel since this amount falls in the linear range of the saturation curve.

For immunoblots, membranes were blocked with 3% dry non-fat milk in Tris-buffered saline plus Tween 20 and probed with 1:500 dilution of the anti-dehydrin antibody (kindly provided by Dr. Close). The bands were detected by alkaline phosphatase assay using Proto Blot Western Blot AP Kit (Promega). The immunoblots were digitally recorded using the Alpha Innotech digital image analysis system and optical density values for the 25 kDa band were recorded using the same system. For optical density determinations, three separate immunoblots for each species were scanned.

Results

Association of 25 kDa dehydrin with CA in a wide array of *Rhododendron* species

The 25 kDa dehydrin was detected both in non-acclimated and cold acclimated leaves of all but one *Rhododendron* species used in this study, with a distinct accumulation to relatively high levels in cold acclimated tissues. In addition to a 25 kDa dehydrin, another 10 dehydrins (ranging from 30 to 73 kDa) were detected in various *Rhododendron* species used in this study. Except for 32, 46 and 73 kDa, all these dehydrins were detected in non-acclimated tissues (Table 1). Similar to 25 kDa, all the other dehydrins accumulated to relatively higher levels in cold acclimated tissues compared to non-acclimated ones (data not shown). Although a 50 kDa dehydrin and a 28 kDa dehydrin were widely observed in cold acclimated tissues of *Rhododendron* species (with their presence in 15 and 6 species, respectively), the 25 kDa dehydrin was the most phylogenetically conserved dehydrin with its presence in 23 of 24 distinct *Rhododendron* species (Table 1).

For a few species used in this study, such as *R. brachycarpum*, *R. dichroanthum*, *R. fortunei*, *R. maximum* and *R. yakushimanum*, several genotypes were employed in the experiments (Table 1). Genotypes within species shared the same dehydrins with one exception - *R. dichroanthum*. For this species the differences between the cultivars employed were significant in that *R. dichroanthum* "Sonata" appeared to accumulate just the 25 kDa protein in response to cold acclimation, while *R. dichroanthum* var. *scyphocalyx* accumulated at least three other dehydrins of higher molecular mass (Table 1). Even if the dehydrins that accumulated in different cultivars were identical, some differences were observed in their relative amount. For example, in *R. yakushimanum*, the cultivar "Koichiro Wada" accumulated the highest amount of 25 kDa dehydrin, while "Mist Maiden" accumulated the highest amount of 50 kDa dehydrin.

The 25 kDa dehydrin was not detected in either non- or cold acclimated leaves of one species: *R. brookeanum* (Table 1). Efforts with even higher loadings of total protein did not reveal this dehydrin on immunoblots (data not shown). In this species, only one dehydrin (approx. 41 kDa) was detected which doesn't appear to be up-regulated in the cold-acclimated samples.

In order to assess the authenticity of the immune signal detected for different bands, as well as for 25 kDa dehydrin, all the blots were incubated with pre-immune serum. The pre-immune serum did not recognize the 25 kDa band and reacted weakly with a band believed to be the major subunit of Rubisco (data not shown).

Cold acclimation ability and 25 kDa accumulation

To investigate the association between the quantitative accumulation of 25 kDa dehydrin and the degree of leaf freezing tolerance (LFT) of different *Rhododendron* species, three 'super' hardy (*R. catawbiense*, *R. maximum* and *R. metternichii*) and three 'less' hardy (*R. arboreum*, *R. dichroanthum* and *R. vernicosum*) species were compared. Immunoblots for each species were run in three replications and the optical density values of the 25 kDa band were recorded for non- and cold acclimated leaves (Fig. 2). No correlation was evident between the absolute amount of 25kDa dehydrin in cold acclimated leaves and their respective LFT (expressed as T_{max}). For example, in *R. maximum* ($T_{max} = -52^{\circ}\text{C}$) the OD value of the 25 kDa band from cold acclimated leaves was ~ 39.4 (Fig. 2A), while for the 25 kDa homologous band of *R. arboreum* leaves ($T_{max} = -20^{\circ}\text{C}$) this value was 49.8 (Fig. 2B).

However, our data indicate that the super hardy species display a higher fold increase in 25 kDa dehydrin during cold acclimation (Fig. 2). For the super hardy species, the average increase in LFT from non-acclimated to cold acclimated stage was about 7.5 fold, which is paralleled by an increase of $\sim 125\%$ in the accumulation of 25 kDa dehydrin. For the less hardy species however, the average increase in LFT was only about 3.2 fold during cold acclimation, while the percent increase in the 25 kDa dehydrin accumulation was $\sim 55\%$. During our experiment with the six *Rhododendron* species (Fig. 2), the 25 kDa dehydrin was not detected in the non-acclimated samples of *R. metternichii*. This appears to be due to exceedingly low levels of this protein in non-acclimated samples of this species since experiments with loadings higher than $7\ \mu\text{g}$ protein made it possible to visualize this dehydrin even in non-acclimated samples of these species (data not shown).

25 kDa dehydrin survey of *Ericaceae* family

Five *Ericaceae* genera, other than *Rhododendron*, were used in this investigation and leaves of non- and cold acclimated plants were used to study the presence/absence of 25 kDa dehydrin in leaf tissues. Our results indicated that the 25 kDa dehydrin was present and cold-induced only in *Kalmia latifolia* (mountain laurel), whereas the other four genera accumulated dehydrins of different molecular weights in response to cold acclimation, but had no homologous 25 kDa bands (Fig. 4A).

Discussion

Association of 25 kDa dehydrin with CA in a wide array of *Rhododendron* species

Plant cold hardiness is a physiologically complex trait that involves the interaction between several biochemical, physiological and morphological factors. It has been suggested that the presence of dehydrins alone is not sufficient to confer tolerance to different types of environmental stresses and that dehydrins work in conjunction with other metabolites (carbohydrates and other cryoprotectants) to reduce freezing stress (Close 1996). Accumulation of the 25 kDa dehydrin in rhododendrons is undoubtedly only one of the several factors responsible for the CA process. However, a critical physiological role for the 25 kDa dehydrin in *Rhododendron* is suggested by the fact that this protein is present in and accumulates during CA in almost all the species used in this study. These species are very diverse, and can be grouped into two subgenera (*Hymenanthus* and *Rhododendron*) that diverged millions of years ago (Table1). This suggests that the 25 kDa dehydrin is a highly conserved dehydrin in *Rhododendron* genus. Additionally, some other species-specific dehydrins were only observed in few genotypes and none exhibited the same consistency of accumulation during CA as displayed by 25 kDa dehydrin across such a wide array of *Rhododendron* species (Table1).

It is interesting to note that in *R. brookeanum*, a species of tropical origin, the 25 kDa dehydrin was completely absent. *Rhododendrons* that originate in tropical regions are not expected to have much ability to cold acclimate unlike their temperate relatives. Our data

indicate that the cold acclimated leaves of *R. brookeanum* had a T_{max} of only -6.9 °C (Table1). Other studies performed with *Rhododendron* species that belong to the same section noted that these species were injured by slight freezing (-3 to -6°C) even after hardening at 0° to 5°C (Sakai et al. 1986). These observations suggest that tropical *Rhododendrons* lack the ability to cold acclimate to any significant level. Our results on the close association of 25 kDa dehydrin accumulation with cold acclimation in a wide array of *Rhododendron* species, and its absence in *R. brookeanum*, collectively suggests that this dehydrin may be of key importance to cold acclimation in *Rhododendron* species. It is also noteworthy that the only dehydrin detected in *R. brookeanum* (41 kDa) doesn't appear to be up-regulated by low temperatures. This might be a constitutive dehydrin that plays no role in the cold acclimation process but may have other "house-keeping" functions.

Only one species (*R. brachycarpum*) failed to fit the general accumulation pattern for 25 kDa dehydrin in cold acclimated leaves. The amount of 25 kDa in non-acclimated samples of this species was found to be higher than that in the cold acclimated leaves. However, all the other dehydrins accumulated at relatively higher levels (compared to non-acclimated tissues) in cold acclimated leaves of this species (data not shown). The apparent anomaly regarding 25 kDa dehydrin accumulation pattern may be due to a possible exposure of non-acclimated samples to other stressful factors (such as dehydration). Considering that the 25 kDa dehydrin may also be induced by water stress, accumulation of this dehydrin to unusually high levels in non-acclimated plants may lead to a biased (erroneous) conclusion regarding its up-regulation during CA, although this was not confirmed.

Dehydrins variability in *Rhododendron* genus

Our data are consistent with previous studies (Close et al., 1993) that dehydrins belong to a highly variable multigene family. The majority of the cold acclimated species used in our study accumulated also some other dehydrins (ranging from 1 to 5) along with the 25 kDa dehydrin. These dehydrins were usually of higher molecular weight (30-73 kDa), and were not conserved across different species with the same consistency as the 25 kDa dehydrin. It is possible that these species also have dehydrins smaller than 25 kDa, but because the smallest marker used for

immunoblots was 20.9 kDa and present at the bottom edge of the gels, we were unable to detect dehydrins smaller than 20 kDa.

Our data indicated a significant intrageneric (interspecific) dehydrin variability among different species of *Rhododendron*. Although intergeneric and intraspecific (varietal or cultivar level) variability of dehydrins in plants has been documented (Ashgar et al. 1994; Guy et al. 1994; Muthalif and Rowland 1994; Sarhan et al. 1997; Wisniewski et al. 1996), data on intrageneric variability in herbaceous and woody species is scarce. The presence of 11 dehydrins distributed among various species of *Rhododendron* suggests that intrageneric differences in cold acclimation may be associated with both quantitative and qualitative differences in dehydrins. In general, little or no intraspecific variability in dehydrins was noted in our study of different cultivars. *R. dichroanthum* was the only species that exhibited intraspecific variability of dehydrins (compare *R. dichroanthum* var. *scyphocalyx* with *R. dichroanthum* "Sonata" in Table 1) which might be due to significant genotype differences since "Sonata" is a hybrid with heterogeneous parentage .

Apparently, having more dehydrins does not necessarily translate into more cold hardy *Rhododendrons*, as evident in Table 1. For example, a super-hardy species (*R. catawbiense*) accumulated only one dehydrin in cold acclimated tissues, whereas other equally hardy species (*R. maximum*) or significantly less hardy species (*R. dichroanthum*) contained several dehydrins. This suggests that not all dehydrins are involved in the cold acclimation process *per se* or their relative contribution to cold hardiness is variable. It is also possible that if these dehydrins contribute to cold hardiness in an additive manner, their synergism varies among genotypes.

Cold acclimation ability and 25 kDa accumulation

Results from our study could not establish a direct correlation between the degree of LFT and the absolute amount of 25 kDa dehydrin in cold acclimated tissues ($R^2 = 0.4$). This raises a question as to how the 25 kDa dehydrin accumulation is significant for *Rhododendron* winter survival. It has been suggested that the super hardy species have a higher ability to withstand low winter temperatures compared to less hardy species, not only due to their genetic background but also due to their higher ability to cold acclimate (Lim et al. 1998 b; Stone et al. 1993).

We compared the amount of 25 kDa dehydrin accumulated in non-acclimated and cold acclimated leaves of 'less' hardy *Rhododendron* species with those found in 'super' hardy species. Our results indicate that the cold inducibility, i.e. increase in the accumulation of this dehydrin in cold acclimated tissues relative to non-acclimated ones (Fig. 2), averaged 55 % in tender species compared to 125 % in super hardy species which parallels ~ 2X higher ability of super hardy species to cold acclimate, as also evidenced in Fig. 2. Previous research in our laboratory demonstrated that mid-winter freezing tolerance in *Rhododendron* was primarily dependent upon the acclimation ability rather than the constitutive LFT and suggested that few 'major' genes control this trait (Lim et al. 1998b). It is possible that 25 kDa dehydrin is the product of one of these major genes, however, genetic mapping studies will be needed to substantiate or refute this notion. Data on the correlation between quantitative accumulation of dehydrins and the level of cold hardiness across diverse species are scarce. Arora et al. (1997) noted that hardy genotypes of blueberry accumulated higher levels of dehydrins compared to the tender ones, showing a positive correlation between the amount of dehydrins and level of cold hardiness. However, this is the first report, to our knowledge, demonstrating a strong correlation (Fig. 3) between cold acclimation ability and cold-inducibility of a specific dehydrin.

25 kDa dehydrin survey of *Ericaceae* family

Investigation of non-acclimated and cold acclimated leaves of mountain laurel (*Kalmia latifolia*) showed that the 25 kDa dehydrin was present in this species and exhibited the same accumulation profile during cold acclimation as in most other *Rhododendron* species. A modified cladogram (adapted from Kron 1997) of *Ericaceae* family members used in this study illustrates a close phylogenetic relationship between *Kalmia* and *Rhododendron* (Fig. 4B). These two species share the same habitat in some regions of North America and there is evidence in literature that *Rhododendron* and *Kalmia* are inter-fertile (Jaynes 1975). The other species, even though related to *Rhododendron*, don't share the same level of similarity, and also lack the 25 kDa dehydrin. Maybe the parallel evolution under identical ecological conditions maintained the 25 kDa protein functional in *Rhododendron* and *Kalmia* species as a low temperature survival mechanism. In those *Ericaceae* species that lacked the 25 kDa dehydrin, accumulation of

different dehydrins as well as other survival mechanisms may play a role in low temperature tolerance.

Concluding remarks

Lim et al. 1999 suggested that the presence or absence of 25 kDa dehydrin could serve as a genetic marker to distinguish between super hardy and less hardy *Rhododendron* species. The presence/absence hypothesis was based on their observation that the 25 kDa dehydrin was absent in *R. fortunei* (one of the parental genotypes used in their experiments). However, with the improved protein extraction protocol used in the present study we were able to identify the 25 kDa dehydrin in non- and cold acclimated leaves of *R. fortunei*, although at significantly lower levels than in *R. catawbiense*. Therefore, it appears that differential accumulation of 25 kDa dehydrin (and perhaps differential hardiness) in *R. catawbiense* and *R. fortunei* may be due to differential gene regulation of 25 kDa dehydrin rather than structural gene differences as previously thought. Based on the data from this study, we now believe that the relative cold-inducibility of this dehydrin may be a 'marker' of cold acclimation ability in *Rhododendron*. Since 25 kDa dehydrin appears to be highly conserved dehydrin among a wide array of *Rhododendron* species, it is cold induced and since its metabolism appears to be tightly linked to cold acclimation ability in *Rhododendron*, an in-depth characterization of this protein is warranted. Cloning the sequences that encode the 25 kDa dehydrin and performing *in vitro* functional (cryoprotection and/or chaperone) assays with purified 25 kDa dehydrin might provide important clues for its role in cold hardiness. Currently, work is also under way in our laboratory to study the environmental regulation (photoperiod, water stress) of this dehydrin in *Rhododendron*.

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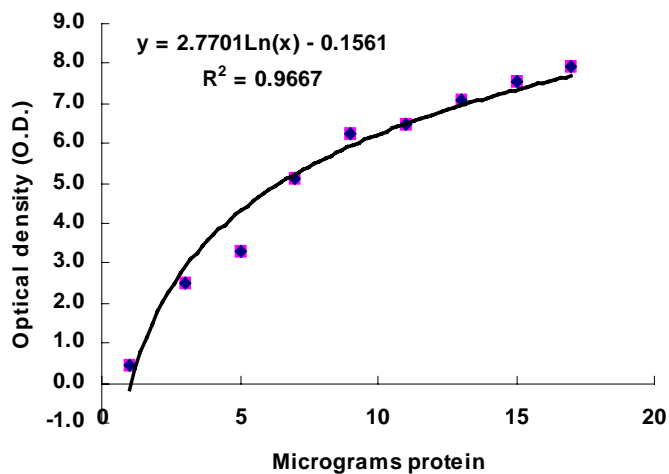
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Table 1. *Rhododendron* species used in this study, their corresponding leaf hardiness and various dehydrin species that accumulate in cold acclimated tissues

No.	Species	Leaf freezing tolerance (LFT)	Dehydrins (kDa)
Subgenus Hymenanthes			
1.	<i>R. adenophorum</i> (=adenogynum)	-29 ^a	25, 50
2.	<i>R. arboreum</i>	-20 ^a	25, 50
3.	<i>R. brachycarpum</i>	-60 ^b	25, 28, 46**, 50, 64
4.	<i>R. brachycarpum</i> var. tigerstedtii	ND	25, 28, 46**, 50, 64
5.	<i>R. brachycarpum</i> "Roslyn form"	ND	25, 28, 46**, 50, 64
6.	<i>R. catawbiense</i> "Catalgla"	-53 ^a	25
7.	<i>R. catawbiense</i> X <i>R. fortunei</i> hybrid "Ceylon"	-43 ^a	25, 41, 46**, 50, 64
8.	<i>R. dauricum</i>	-50 ^b	25, 30, 50
9.	<i>R. decorum</i>	ND	25, 28
10.	<i>R. dichroanthum</i> var. scyphocalyx	-23 ^a	25, 28, 30, 32**
11.	<i>R. dichroanthum</i> "Sonata"	ND	25
12.	<i>R. fargesii</i>	ND	25, 50, 64
13.	<i>R. fortunei</i> "Gable"	-38 ^a	25, 28, 41, 46**, 50, 64
14.	<i>R. fortunei</i> no. 27 "Dexter"	ND	25, 28, 41, 46**, 50, 64
15.	<i>R. makinoi</i>	ND	25, 30, 41, 50
16.	<i>R. maximum</i>	-52 ^a	25, 30, 50, 73**
17.	<i>R. maximum</i> var. rubrum	ND	25, 30, 50, 73**
18.	<i>R. maximum</i> "Mt. Mitchell"	ND	25, 30, 50, 73**
19.	<i>R. metternichii</i>	-48 ^a	25, 28, 50
20.	<i>R. vernicosum</i> "Gable's vernicosum"	-25 ^a	25, 28, 37, 50
21.	<i>R. yakushimanum</i>	-40 ^b	25, 50
22.	<i>R. yakushimanum</i> "Koichiro Wada"	ND	25, 50
23.	<i>R. yakushimanum</i> "Mist Maiden"	ND	25, 50
Subgenus Rhododendron			
24.	<i>R. brookeanum</i>	-7 ^a	41*
25.	<i>R. dauricum</i>	-50 ^b	25, 30, 50
26.	<i>R. hirsutum</i>	ND	25, 37
27.	<i>R. keiskei</i> "Mt. Kuromi"	-25 ^b	25, 37
28.	<i>R. minus</i>	ND	25, 30, 50, 64
29.	<i>R. mucronulatum</i>	-50 ^b	25, 34, 50
30.	<i>R. myrtifolium</i>	ND	25
31.	<i>R. russatum</i>	-40 ^b	25, 32**, 41

^a LFT estimated in our laboratory and expressed as T_{max} (the temperature causing maximum rate of injury);
^b Sakai et al. 1986 - LFT expressed as LST (the lowest survival temperature);
*does not increase in cold acclimated leaves compared to the non-acclimated leaves;
** not detected in non-acclimated leaves; ND not determined.

A



B

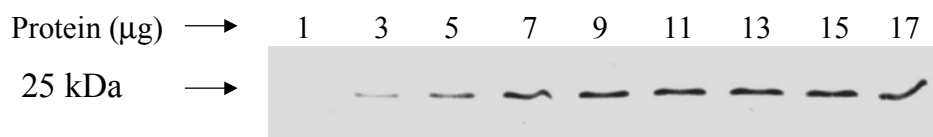


Figure 1. A. Saturation curve for anti-dehydrin immunoblot of *Rhododendron* (*R. catawbiense*) leaf proteins. Optical density (O.D.) values of 25 kDa dehydrin band were plotted against different amounts of total protein **B.** Anti-dehydrin immunoblot profile of 9 different loadings of total protein extracted from cold acclimated leaves of *Rhododendron*. The protein amounts and 25 kDa dehydrin are indicated by arrows.

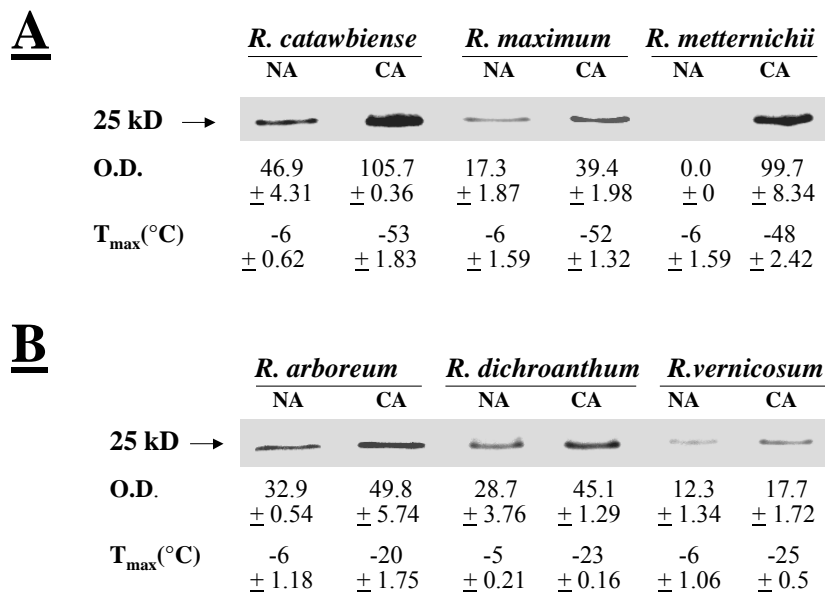


Figure 2. Anti-dehydrin immunoblots for the 25 kDa dehydrin (indicated by arrows) of leaf proteins from three super hardy (**A**) and three less hardy (**B**) *Rhododendron* species. All lanes were loaded on an equal protein basis (7µg/lane). The optical density for the 25 kDa dehydrin band and the T_{max} (temperature causing maximum rate of injury) value for non- and cold acclimated leaves of each species are indicated. O.D.-optical density. NA-non-acclimated, CA-cold acclimated.

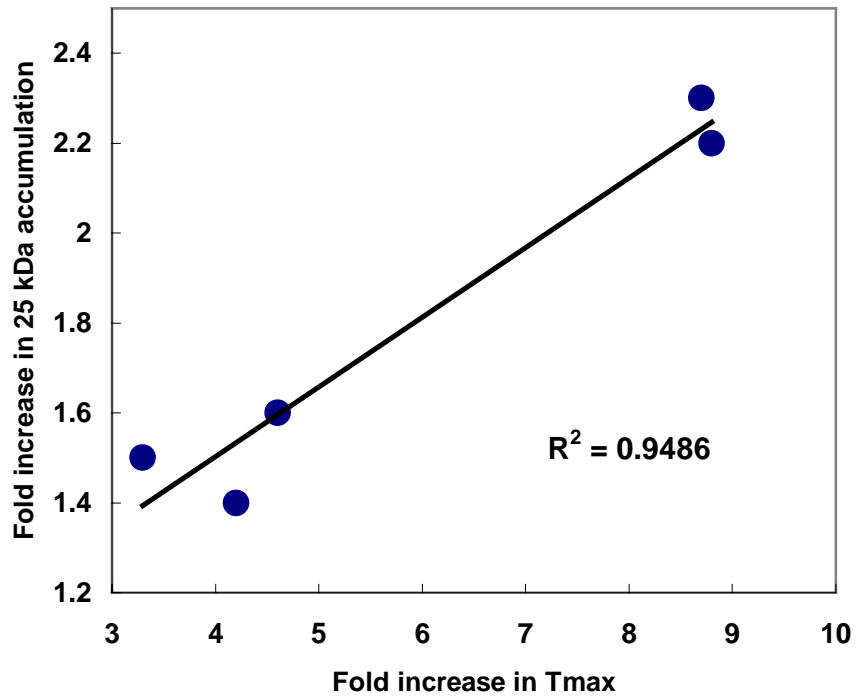


Figure 3. Relationship between cold acclimation ability (fold increase in T_{\max} - temperature causing maximum rate of injury) and 25 kDa dehydrin accumulation (fold increase expressed as optical density values) in five *Rhododendron* species (*R. metternichii* omitted). R^2 - correlation coefficient

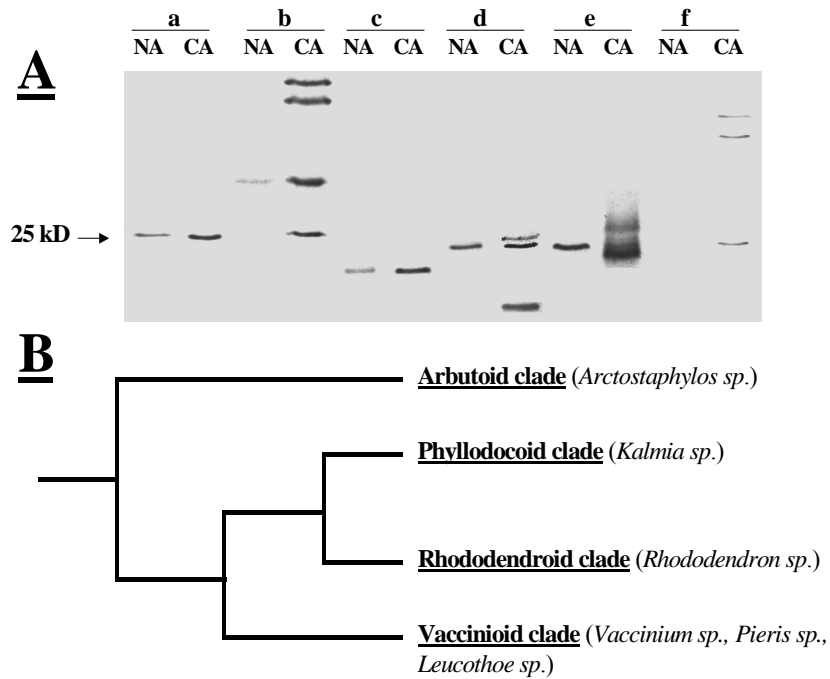


Figure 4. A. Anti-dehydrin immunoblot profile of leaf proteins from six Ericaceae species a) *R. catawbiense*, b) *Kalmia latifolia*, c) *Vaccinium macrocarpon*, d) *Leucothoe fontanesiana*, e) *Pieris floribunda*, f) *Arctostaphylos uva-ursi*. The 25kDa dehydrin is indicated by an arrow. **B.** Cladogram representing phylogenetic relationships within *Ericaceae* family (adapted from Kron 1997). All lanes were loaded on an equal protein basis (7µg/lane). CA-cold acclimated, NA-non-acclimated.

CHAPTER THREE: ENVIRONMENTAL REGULATION AND TISSUE LOCALIZATION OF 25 kDa DEHYDRIN IN RELATION WITH COLD HARDINESS IN *RHODODENDRON*

Abstract

The influence of day-length and temperature on the accumulation of 25 kDa dehydrin and cold acclimation in *Rhododendron* cv. "Chionoides" was studied by growing four groups of plants under different photoperiod and temperature regimes. Combination of short days / low temperature induced the greatest cold hardiness and 25 kDa accumulation, while exposure to long days and relatively high temperatures failed to induce any significant cold tolerance in leaves. Shortening day-length is sufficient to trigger both the first stage of cold acclimation and 25 kDa dehydrin induction, however, low temperatures prevalent later in the Fall and winter caused more pronounced increase in both hardiness and dehydrin accumulation. We found that the water content of plant leaves maintained under natural photoperiod was lower than that of plants grown under extended photoperiod, regardless of the temperature regime. It is hypothesized that early 25 kDa dehydrin accumulation may be due to short-day-induced cellular dehydration. This dehydrin is also induced by water stress, while the exogenous abscisic acid (ABA) application failed to trigger its accumulation. Tissue localization studies show that the 25 kDa dehydrin accumulates more in the leaf lamina than in the midrib. Lower accumulation in the midrib is perhaps associated with a higher incidence of freeze injury in midrib vs. lamina tissues of *Rhododendron* leaves.

Key words: abscisic acid, cold acclimation, dehydrins, photoperiod, *Rhododendron*, water stress

Introduction

Most temperate perennial plants undergo a transition from cold-sensitive to cold-hardy status during the Fall. This transition, known as cold acclimation (CA) reaches a maximum in the winter. A number of morphological and biochemical changes are associated with the induction of freezing tolerance during CA. The study of this phenomenon might help decipher the intrinsic physiological mechanisms that occur during CA in plants and provide valuable information to both the ornamental plants industry and plant breeders interested in improved plant cold hardiness.

Apparently, cold acclimation in woody perennials occurs in at least two stages (Levitt 1980). The first stage is induced by increasingly short daylengths in the fall whereas the second stage is induced by low but above freezing temperatures (Arora et al. 1992). Biochemical and physiological changes associated with these two stages of cold acclimation are not well understood. There also appears to be a third stage of cold acclimation whereby sub-freezing temperatures induce a further increase in freeze tolerance that culminates in fully cold acclimated state (Weiser 1970).

In *Rhododendrons*, short-day photoperiod and low temperatures are believed to interact in order to induce maximum cold tolerance (Cameron and Dixon 2000; Vainola et al. 1999). Combination of short days and low temperatures resulted in the greatest cold tolerance, while exposure to long days and relatively high temperatures reduced the cold tolerance of leaves. However, it was reported that cultivars may differ in their response - some benefitting equally from short days and low temperatures while other being less sensitive to photoperiod but attaining better hardiness when exposed to low temperatures (Vainola et al. 1999). Research performed in *Rhododendron* corroborate with previous findings in the genus *Picea*, where low temperatures combined with short days rapidly increased cold tolerance (Christersson 1978). However, some deciduous species, such as *Alnus*, require just exposure to low temperatures (Tremblay and Lalonde, 1987), while other deciduous plants, such as *Cornus* are sensitive to photoperiod as well as temperature (Harrison 1978). While in deciduous species seasonal cold acclimation is synchronous with dormancy transitions, in over-wintering leaves of *Rhododendron* this phenomenon is easier to study due to its evergreen character.

Several proteins have been identified to accumulate in plants in response to any environmental stimulus that has a dehydrative component. This includes drought, low temperature and salinity. Some of the proteins that are induced during CA include cryoprotectins (Sieg et al. 1996), anti-freeze proteins (Griffith et al. 1997) and Group 2 LEA (late embryogenesis abundant) proteins, also called dehydrins (Close et al. 1993). Dehydrins are characterized by a consensus 15 amino acid sequence EKKGIMDKIKEKLPG that is present near the carboxy terminus, and in additional copies upstream of the terminus, in many cases as a slightly modified 14 amino acid consensus KKGIKEKIKEKLPG (Close et al. 1993). It is believed that dehydrins, having a hydrophilic nature (thereby protecting macromolecular structures from desiccation) are cryoprotectants with detergent and chaperone-like properties (Lin and Thomashow 1992; Close 1996; Wisniewski et al. 1999). However, there is limited understanding of the environmental regulation (photoperiod and low temperature) of dehydrins during seasonal development of freeze tolerance in woody perennials. Particularly, it is unknown if the accumulation of dehydrins in *Rhododendron* is induced by short daylength, low temperatures or both.

Our previous research has demonstrated the importance of a 25kDa dehydrin in cold acclimation of *Rhododendron* species. Lim et al., 1999 indicated that in cold acclimated plants of segregating F₂ progeny, the levels of a 25 kDa dehydrin were closely associated with differences in leaf freezing tolerance. Moreover, differential accumulation of the 25 kDa protein among *Rhododendron* genotypes significantly affected cold hardiness status suggesting that this dehydrin may be a genetic marker for cold hardiness. More recently, the accumulation of this dehydrin during cold acclimation was consistently observed in a wide array of *Rhododendron* species (Chapter two). It was further suggested that the accumulation of 25 kDa dehydrin is correlated with the cold acclimation ability of *Rhododendron* species.

Here we report the effect of environmental factors (photoperiod and temperature) on the accumulation of 25 kDa dehydrin in relation with cold acclimation in *Rhododendron*. Experiments were also conducted to study its induction by water stress and abscisic acid (ABA). Furthermore, accumulation pattern of this dehydrin in different plant tissues in response to cold acclimation and water stress was determined.

Materials and methods

Plant material

Five-year-old clones of the *Rhododendron* cultivar 'Chionoides' (a *R. ponticum* hybrid) were maintained in 5 gallons pots with artificial mix (1:1:1 pine bark, perlite and sphagnum peat) under natural conditions (outdoor location). The plants were periodically fertilized with Azalea Special 21-7-7 (W.R. Grace, Fogelsville, PA, USA) at 1.5 gL⁻¹ plus Fe chelate (Sequestrene 330 Fe, 10% Fe, Ciba-Geigy, Greensboro, NC, USA) at 0.25 gL⁻¹ to maintain pH at 4.5-5.5 and electrical conductivity (EC) at 0.5-0.9 dSm⁻¹ of the potting medium. Plants were watered as needed prior to being subjected to different treatments.

Photoperiod experiment

The experiments were initiated at the end of August. Four different treatments were employed in order to assess the influence of photoperiod and temperature on cold acclimation. Two groups of 6 plants each were kept in the greenhouse, exposed to daily average temperatures of 20-24°C, while the other two groups of 6 plants each were maintained outside, under natural temperature conditions. One treatment of each group (outside and inside the greenhouse) was supplemented with artificial light (~10 μEm⁻²sec⁻¹ at canopy level) provided by incandescent bulbs (Sylvania 75W, GTE Corp. Salem, MA, USA) mounted on a wood frame. Supplemental light treatment began in the first week of September and maintained 15 D / 9 N photoperiod throughout the course of study. This type of treatment did not influenced the temperature of leaves, as monitored daily at all four experimental locations. Monthly samples of randomly collected leaves across all 6 plants/treatment were obtained between September 5 until January 5 to be used for cold hardiness, dehydrin profiles and other experiments described below. Leaf tissues for dehydrin analysis were ground immediately in liquid nitrogen and stored at -80°C to be used later.

Water content

Duplicate samples of leaves from each treatment at each sampling date were dried at 80°C for 48 h (or until constant dry weight was achieved). The water content was expressed as a percentage on dry weight basis.

Water stress experiment

Six separate potted plants of *R. "Chionoides"* were brought in the greenhouse (in July) and water stress was administered on 3 plants by withholding irrigation for three weeks, or until moderate wilting was visible. The other three plants were watered to saturation on a regular basis to serve as controls.

Abscisic acid (ABA) treatments

Experiments were conducted (with a separate set of four plants maintained in the greenhouse during July) to determine if the 25 kDa dehydrin is induced by exogenous ABA treatments in *Rhododendron*. Preliminary experimental protocol consisted of spraying the leaves and watering the pots daily with 50 µmol/L ABA for two weeks. Other two plants were sprayed with -ABA solution and served as control. Since no significant difference in leaf freezing tolerance (LFT) or 25 kDa dehydrin profiles were detected following this treatment, an alternate protocol was used to better ensure the uptake of ABA by leaves. It consisted of placing excised leafy twigs in Erlenmeyer flasks with 100 µmol/L ABA solution. The stems were cut under solution periodically and leaf transpiration was enhanced by blowing gentle air via a fan in order to facilitate uptake of ABA solution into twigs. The Erlenmeyer flasks were covered with aluminum foil to prevent photo-degradation of ABA and were sealed with laboratory film to prevent solution evaporation, allowing only the end of the twigs to protrude. An appropriate control was included in this experiment.

Relative cold hardiness estimation

Leaf freezing tolerance (LFT) was determined through a controlled-freezing protocol in the laboratory and injury was assessed by ion-leakage from leaf tissues. Cooling rates, ion leakage calculations, % injury estimations, Gompertz functions fitting, determination of T_{max} (temperature causing maximum rate of injury and defined as leaf freezing tolerance) and statistical analysis were performed as described by Lim et al. 1998a.

Total protein extraction and estimation

Protein were extracted from 2g of plant tissue with polyvinylpolypyrrolidone (0.8 g) in 11 ml borate buffer (50mM sodium tetraborate, 50mM ascorbic acid, 1mM phenylmethylsulfonyl fluoride, pH 9.0). Crude extracts were shaken on a gyratory shaker at 4 °C for 30 min followed by centrifugation at 26,000 g for 1.5 h at 4°C. Supernatant (soluble proteins) was collected and filtered through 0.45 µm filters. Proteins were then precipitated with 10 % of extract volume trichloroacetic acid and centrifugation at 14,000 rpm for 20 minutes at 4 °C. The protein pellets were washed three times with cold acetone. After each acetone wash a sterile sealed pipette tip was used to physically break the pellet. Dried protein pellets were re-hydrated with 100 µl of 0.5 x sample solubilization buffer (Owl Separation Systems, 0.125 M Tris-HCl pH 6.8, 1% sodium dodecyl sulfate, 5% β-mercaptoethanol, 15% glycerol, 0.005 % Bromphenol Blue) followed by boiling on a water bath for 5 minutes. Vigorous vortex for 30 min was followed by centrifugation at 14,000 g for 5 minutes in order to precipitate the non-protein material.

The Esen method (1978) for determining total protein content in the sample buffer dissolved proteins was used, as described by Lim et al. 1999.

SDS-PAGE and immunoblotting

For immunoblots, 7µg of protein were separated by discontinuous SDS-PAGE with a PROTEAN II electrophoresis unit (Bio-Rad) using 4% stacking gel and 12.5 % running gel and transferred to 0.45 µm nitrocellulose membranes using a Mini Trans-Blot Electrophoretic

Transfer Cell (Bio-Rad) equipped with a cooling unit. Electroblothing was carried out for 1 1/2 hours at 100 V in blotting buffer (25mM Tris-HCl, 192 mM Glycine, 20 % methanol, pH 8.3). Membranes were blocked with 3 % dry non-fat milk in Tris-buffered saline plus Tween 20 (10mM Tris-HCl pH 8, 150mM Sodium chloride, 0.05 % Tween 20) and probed with 1:500 dilution of the antibody directed against a synthetic peptide of the 15 amino acid consensus sequence (EKKGIMDKIKEKLPG) that is highly conserved at the C terminus of dehydrin proteins from several plant species (kindly provided by Dr. Close). The bands were detected by alkaline phosphatase assay using Western Blot AP Kit (Promega). The immunoblots were digitally recorded using the Alpha Innotech digital image analysis system.

Results

***Rhododendron* cold hardiness under various photoperiod and temperature regimes**

Data indicate that only two treatments that were maintained under natural photoperiod (indoor and outdoor) registered a significant increase in cold hardiness by October 5 sampling during the first month (Fig. 1). The treatments supplemented with artificial light did not show any increase in hardiness, regardless of temperature regime during this period (Fig. 1).

By November 5th, both lighted and non-lighted groups of plants maintained outside exhibited a significant increase in cold hardiness, while either group of plants maintained in the greenhouse did not display significant increase in their cold tolerance after October 5 sampling (Fig. 1). Both plant groups maintained outside further increased their hardiness through January 5th sampling at which time the leaf freezing tolerance (LFT) of the lighted and non-lighted plants were statistically similar.

Of all the four treatments, the plants exposed to extended daylength, in the greenhouse did not cold acclimate at all to any significant level during the winter, while the plants exposed to outdoor temperatures registered the most significant increase in cold hardiness (Fig. 1). Samples maintained in the greenhouse, under natural photoperiod, attained an intermediate cold hardiness (Fig. 1).

Water content

For the treatments maintained outside, the plants exposed to natural photoperiod maintained significantly lower leaf water content than the plants exposed to extended daylength throughout the course of study (Table 1). Despite the fact that plants maintained in the greenhouse did not exhibit significant differences in the leaf water content, it was slightly higher in plants maintained under extended daylength than their natural photoperiod-exposed counterparts. No consistent seasonal trend (increase or decrease) in leaf water content was observed during the course of study in different treatments.

25 kDa accumulation in response to photoperiod and temperature

The 25 kDa protein was present in all the samples analyzed (Fig. 2A & 2B). By October 5th a significant accumulation of 25 kDa dehydrin was already observed in the plants maintained outside, under natural photoperiod, compared to the plants exposed to artificial extended daylength, where this accumulation was not evident (Fig. 2A). During the same sampling interval (September 5 - October 5), the plants maintained in the greenhouse under natural photoperiod exhibited similar accumulation of 25 kDa, whereas the plants exposed to extended photoperiod did not show any increase in the amount of this dehydrin (Fig. 2B).

Although 25 kDa dehydrin accumulation began to increase in the lighted plants from October 5 samples onwards, throughout the experiment all the plants exposed to extended daylength accumulated less 25 kDa dehydrin compared to the plants exposed to natural photoperiod regardless of the temperature regime. Moreover, in the plants maintained outside (natural temperatures), the accumulation of other two dehydrin-like proteins of higher molecular weight (approx. 26 and 32 kDa) was observed (Fig. 2A). The 26 kDa band is visible only in the *Rhododendron* leaves collected in January whereas 32 kDa dehydrin was detected in both December and January samples. These two dehydrins were not detected in the greenhouse-maintained plants (Fig. 2B).

Water stress and cold induction of 25 kDa dehydrin and its tissue localization

The *Rhododendron* cultivar 'Chionoides' appears to be very resistant to extended water stress. Only after three weeks of withholding watering the plants developed wilting symptoms. The anti-dehydrin immunoblots clearly show that the 25 kDa dehydrin is induced by water stress (Fig. 3 A). Two additional dehydrins (approx. 26 and 32 kDa) were also observed to accumulate in response to this treatment. These dehydrins were the same as ones identified in the December and January outdoor samples (Fig. 2A).

The 25 kDa dehydrin appears to be present in the leaves, buds and bark of water stressed (Fig. 3A) and cold acclimated (Fig. 3 B) *Rhododendron* plants. However, this dehydrin was not detected in the xylem of cold acclimated plants (Fig. 3B). Regardless of the environmental cue (low temperature or water stress), accumulation of 25kDa dehydrin occurs mostly in the leaf lamina, while significantly lower levels (compared to lamina) were detected in the midrib (Fig. 3A and 3B).

ABA induction of 25 kDa dehydrin

Immunoblots showed that both ABA treatments failed to induce the accumulation of 25 kDa dehydrin (data not shown). When the first treatment was employed (spraying and watering with ABA solution) no visual control was available to insure the ABA uptake into the plant. However, with the set-up involving excised twigs in ABA solution, significant uptake of ABA solution in the twigs was visually observed. No increase in leaf freezing was observed in ABA treated leaves compared to control (data not shown).

Discussion

Cold hardiness

Our results confirmed previous observations by Cameron and Dixon (2000) that low temperatures and short photoperiods together lead to maximum cold tolerance in *Rhododendron*

plants. Between September and October the only significant increase in cold hardiness was observed for the plants maintained under natural photoperiod indicating that short photoperiod triggers the first stage of cold acclimation (Fig.1). The daylength reduction from September (12.75 hours) to October (10.5 hours) was perhaps enough to cause an increase in cold hardiness. No significant increase in LFT was noticed in September samples compared to July samples (data not shown) and unfortunately no data could be collected during August. Apparently, certain critical photoperiod is required to trigger the first stage of cold acclimation in *Rhododendron*, the length of which would depend upon geographical location and ecological conditions. For example, Vainolla et al. 1999 reported that even exposure to 14 hours daylength could be perceived as an inductive short day for cold acclimation response in rhododendrons adapted to high latitudes in Finland. Our data however, indicate that daylength less than 12.75 hours may be sufficient to trigger the first stage of cold acclimation in *Rhododendron* "Chionoides".

Between October and November a dramatic increase in hardiness was observed for the plants exposed to extended photoperiod and natural temperature conditions. Although outdoor monthly average temperatures did not register a significant drop (Fig. 4), it is noteworthy that during this period two light frosts occurred on Oct 29 and Nov 6. Sub-freezing temperatures during these frost episodes perhaps induced this sudden increase in cold hardiness. It appears that while short days trigger the cold acclimation process initially, low temperatures can eventually override the photoperiod stimulus. Our data indicate that exposure to short days alone induced a small but significant increase in cold hardiness as evident in the plants maintained in the greenhouse, under natural photoperiod (Fig. 1). This suggests that there is a physiological limit to which *Rhododendrons* can acclimate in response to short photoperiod alone.

The plants that were not exposed to either short days or low temperatures (greenhouse plants under extended photoperiod) did not register any increase in cold hardiness. It is possible that the endogenous circadian rhythms do not play a role in cold acclimation when *Rhododendrons* are maintained under artificial conditions over winter. Previous research indicating that in the absence of short days and low temperatures *Rhododendrons* continue their active growth, without entering dormancy (Doorenbos 1955; Vainola and Juntilla 1998), support this notion. This phenomenon may have practical application in shortening the breeding cycle of this species (hastening the physiological maturity) by maintaining *Rhododendrons* under 24 hours photoperiod (Doorenbos 1955).

Photoperiod and low temperature induction of 25 kDa dehydrin

Our data indicate that the 25 kDa accumulation is triggered by short photoperiod, however, more pronounced increase occurs in response to low temperatures (Fig. 2A). Furthermore, short photoperiods alone are sufficient to trigger its accumulation as observed in greenhouse plants exposed to natural photoperiod (Fig. 2B). The mechanisms for short photoperiod induced accumulation of dehydrins is not yet understood. However, it is believed to involve a phytochrome-mediated response. The question arises - what is the primary signal for this phytochrome-mediated response? It has been shown that cold acclimation promotes a water loss (desiccation) in certain deciduous woody plant tissues (Rinne et al. 1998). It is possible that short daylength during first stage of cold acclimation induces a decrease in leaf water content via a phytochrome-mediated mechanism similar to that found in deciduous woody plants. If the same mechanism functions in *Rhododendron* leaves, then a low water content, perceived as a "dehydration", could be the signal that induces the accumulation of 25 kDa dehydrin. Although data on leaf water content in our study did not indicate a consistent trend, it clearly showed that the leaf water content of plants maintained under natural photoperiod was significantly lower than that in plants exposed to extended daylength (Table 1). This observation is even more significant for plants maintained in the greenhouse under natural photoperiod since they received regular watering.

Interestingly, a significant increase in 25 kDa accumulation during the course of investigation was observed even in plants maintained in the greenhouse under extended photoperiod, although the accumulation was significantly lower than that in other three groups (treatments) of plants which all displayed cold acclimation. Greenhouse plants maintained under extended photoperiod did not display any CA. Therefore the accumulation of 25 kDa dehydrin in these plants cannot be explained in terms of increased cold hardiness. However, it may be a consequence of altered turnover rate of this protein under extended photoperiod and warmer temperatures.

The other two dehydrins identified in cold acclimated samples (26 and 32 kDa) appear to be induced only by low temperatures (Fig. 2A) because they were not identified in the plants maintained in the greenhouse (Fig. 2B). It also appears that sub-freezing temperatures may

trigger the induction of these dehydrins since they accumulate late in the winter (December and January), after temperatures dropped significantly below zero (Fig. 4). These dehydrins may be responsible for the increased hardness of the plants exposed to natural temperatures in an additive manner, since the relative amount of 25 kDa dehydrin present in cold acclimated leaves (both indoor and outdoor) was not significantly different.

Water stress and ABA induction

In order to determine if this dehydrin is specifically regulated by cold or not we studied its induction in response to water stress. Our data indicate that the 25 kDa dehydrin is induced by both water stress and cold. To our knowledge, there is no information available on a direct correlation between cold hardness and drought resistance in *Rhododendron*. However, studies with azalea show that cold acclimation can be induced by withholding watering at the end of the summer (Anisko and Lindstrom 1996). Most likely water stress induces accumulation of several metabolites with cryoprotectant properties (including dehydrins). A similar mechanism may be found in larger *Rhododendron* species. It would be interesting to explore a correlation between drought tolerance and cold hardness in different *Rhododendron* species and to test if accumulation of 25 kDa dehydrin (or other dehydrins) is a physiological link between the two. It is important to note that the other two dehydrins that accumulate in cold acclimated plants (26 and 32 kDa) were induced by water stress too (Fig. 3A). This suggests that these two dehydrins have a similar function and regulation as 25 kDa dehydrin, and probably contribute to the protection of leaves against dehydrative stress.

Several studies have reported the induction of dehydrins by ABA in a wide variety of species (Zhang et al. 1996; Pelah et al. 1997; Eggerton-Warburton et al. 1997). These studies typically used spraying or dipping the aerial parts in different concentrations of ABA solution as treatments. We used two strategies for ABA treatment in *Rhododendron* plants. Even though we did not measure the tissue ABA content after ABA treatment, we believe that ABA was taken up by *Rhododendron* leaves, especially during the excised twig treatment. Although our conclusion is mostly speculative, the results suggest that ABA treatment does not induce CA and 25 kDa dehydrin accumulation in *Rhododendron*. It has been reported that the onset of freezing tolerance in birch (*Betula pendula*) is dependent on endogenous ABA accumulation (Rinne et al. 1998;

Welling et al. 1997) and is impaired to some extent in an ABA - deficient genotype. However, even in the absence of ABA accumulation, the plants developed a certain level of freeze tolerance. Our results suggest that increase in LFT at non-inductive temperatures in *Rhododendron* is not dependent on the endogenous accumulation of ABA.

Tissue localization

Although the 25kDa dehydrin is present in almost all the tissues studied, few exceptions were noted. The dehydrin of interest was not detected in the xylem tissue of cold acclimated plants and buds of water stressed plants. Moreover, the amount of 25 kDa dehydrin in leaf lamina was significantly higher than that found in other tissues (Fig 3A & 3B). If this dehydrin plays a protective role during freezing stress, its accumulation would be expected to correlate with the freeze damage. During winter, two types of injury symptoms can be identified on *Rhododendron* leaves. One is caused by winter desiccation, while the other by freezing (presence of ice in the tissue). We noticed, as have others (Holt and Pellet 1981), that the midrib and veins are more sensitive to freezing injury than the lamina (Fig. 5). Relatively greater freeze-injury of midrib may be due to higher amount of free water present in the leaf vascular tissue. When disruptive ice formation occurs in these tissues, damage and necrosis are visible in the midrib. It is noteworthy however, that 25 kDa was present at exceedingly low levels in midrib tissues (Fig. 3A & 3B). During a frost episode, ice formation in the tissue apoplast creates a vapor pressure gradient between the midrib and lamina tissues. Lamina exposes also a larger surface to the environmental factors compared to the midrib and, thus, is more prone to winter desiccation. Therefore it is expected that a strategy exists to prevent cellular dehydration in lamina tissues and higher accumulation of dehydrins could be one component of that strategy.

Concluding remarks

The results presented here provide important information about the short day - and low temperature - induction of 25 kDa in relation with cold hardiness in *Rhododendron*. Its induction closely parallels the seasonal cold acclimation suggesting an important role of this dehydrin in cold acclimation process. Uniquely enough, this dehydrin is induced by water stress but does not

appear to respond to ABA treatment. Finally, its differential localization in leaf lamina vs. vein tissue may be related to its cryoprotective role during freezing.

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Table 1. Seasonal fluctuations in leaf water content (expressed as % on dry weight basis) of *Rhododendron* cv. "Chionoides" maintained under four different temperature and light regimes

Sampling date	Water content (% dry weight basis)			
	Outdoor-Natural Photoperiod	Outdoor- Extended Daylength	Indoor- Natural Photoperiod	Indoor- Extended Daylength
Sep 5	124.6±3.6	134.3±2.9*	135.8±2.5	143.4±3.8*
Oct 5	132.4±2.7	150.9±0.9	137.9±2.1	129.9±5.7*
Nov 5	127.9±0.4	137.6±2.1*	141.1±0.0	141.3±7.8*
Dec 5	111.3±0.7	123.2±0.0	138.3±2.3	143.2±5.9*
Jan 5	114.7±1.5	130.6±11.8*	138.1±5.7	159.5±4.4

* values significantly different from their natural photoperiod -exposed counterparts at P<0.05 (determined by *t*-test)

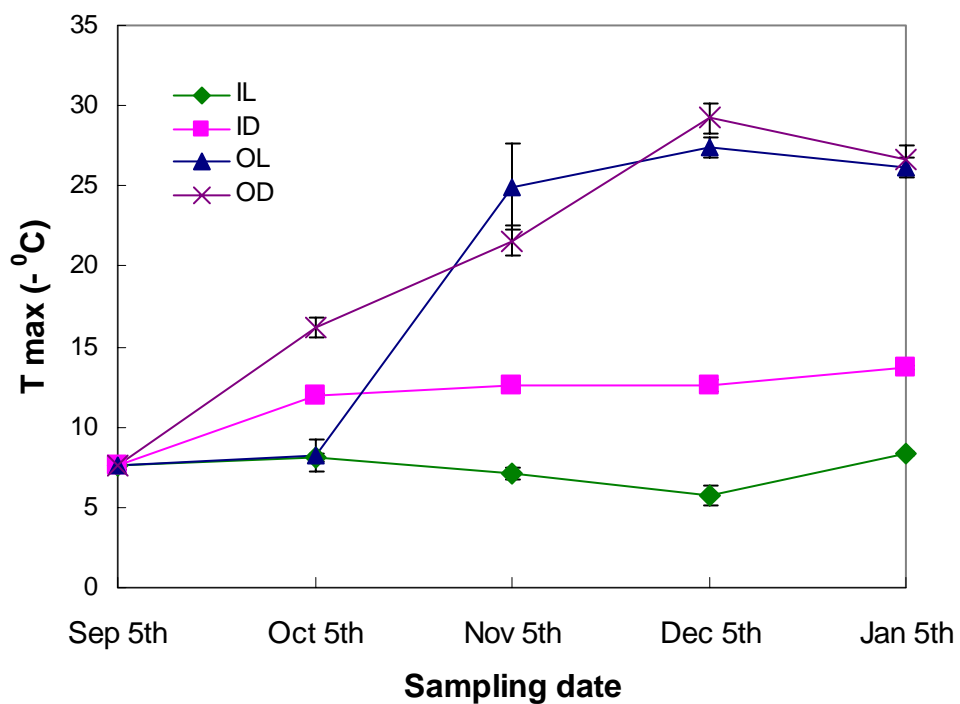


Figure 1. The effect of light and temperature on leaf freeze tolerance (expressed as T_{max} - temperature causing maximum rate of injury) of *Rhododendron* cv. Chionoides. IL-greenhouse temperatures with extended daylength, ID - greenhouse temperatures with natural photoperiod, OL - natural temperatures and extended daylength, OD - natural temperatures and photoperiod.

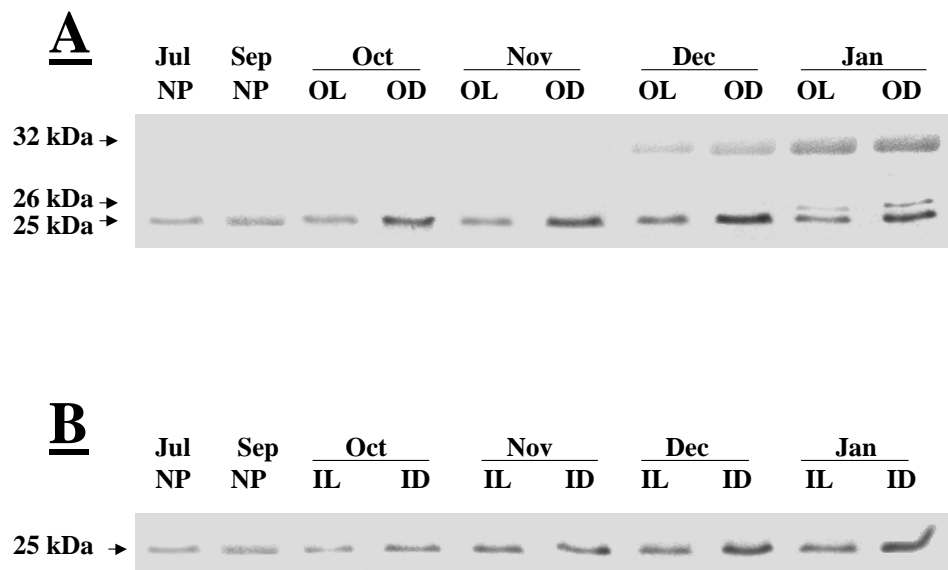


Figure 2. The effect of light and temperature on the accumulation of 25 kDa dehydrin in *Rhododendron* leaves. **A.** Plants maintained under natural temperatures **B.** Plants maintained under greenhouse temperatures. NP - natural photoperiod for controls, IL - greenhouse temperatures with extended daylength, ID - greenhouse temperatures with natural photoperiod, OL - natural temperatures and extended daylength, OD - natural temperatures and photoperiod.

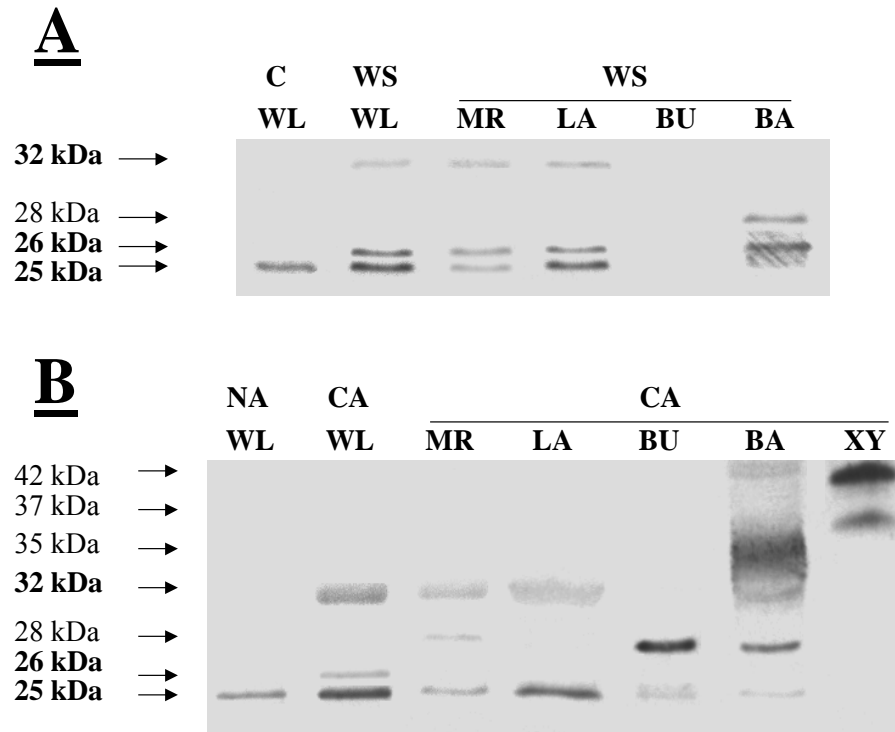


Figure 3. Accumulation of 25 kDa dehydrin in different *Rhododendron* tissues during water stress (**A**) and cold acclimation (**B**). NA - non-acclimated, CA - cold acclimated, C - control, WS - water stressed, WL - whole leaf, MR - midrib, LA - lamina, BU - bud, BA - bark, XY - xylem

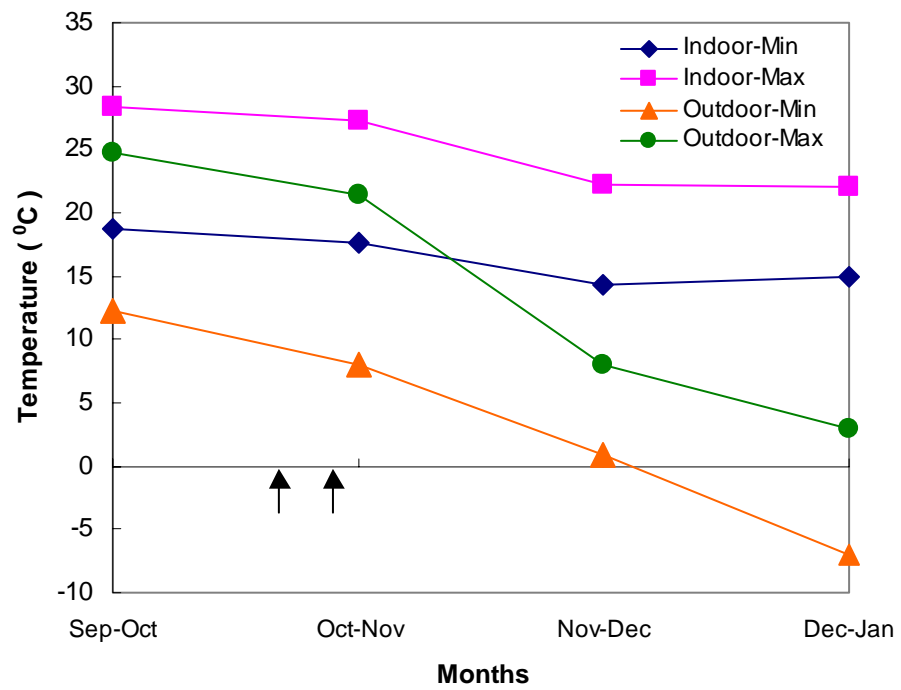


Figure 4. Minimum and maximum average monthly temperatures for greenhouse and outside experiment locations. First two frosts dates are indicated by arrows.



Figure 5. Visual symptoms of freeze injury in the leaves of *Rhododendron* cv. Chionoides.

CHAPTER FOUR: SUMMARY AND CONCLUSIONS

Summary

This research investigated the presence and accumulation patterns of a 25 kDa dehydrin (previously found to play an important role in *Rhododendron* leaf hardiness) and of other dehydrins that accumulate during cold acclimation in a wide array of *Rhododendron* species (24 in total) belonging to two diverse subgenera - *Hymenanthes* and *Rhododendron*. Experiments were also conducted to study whether accumulation levels of 25 kDa dehydrin are associated with cold acclimation ability in *Rhododendron*. A total of 11 cold-induced dehydrins were detected across all 24 species but only the 25 kDa dehydrin was the most conserved across this genus. *R. brookeanum* - a species of tropical origin that lacks cold acclimation ability - was the only species which does not possess this dehydrin. The quantitative accumulation of 25 kDa dehydrin appears to be linked with cold acclimation ability. Investigations with five other genera belonging to *Ericaceae* family (*Kalmia latifolia*, *Arctostaphylos uva-ursi*, *Vaccinium macrocarpum*, *Leucothoe fontanesiana* and *Pieris floribunda*) revealed that the 25 kDa dehydrin was present and accumulated during cold acclimation only in *Kalmia latifolia*, a species most closely related (phylogenetically) to *Rhododendron*.

In order to establish the influence of different environmental factors (photoperiod and temperature) on the accumulation of 25 kDa dehydrin in response to cold acclimation in *Rhododendron* cv. "Chionoides", four groups of plants were grown under different photoperiod and temperature regimes. Combination of short days and exposure to natural low temperatures induced the greatest cold hardiness and 25 kDa accumulation, while exposure to long days and relatively high temperatures failed to induce any significant cold tolerance in leaves. Short day-lengths are sufficient to trigger both the first stage of cold acclimation and 25 kDa dehydrin induction but, later in the Fall and winter, low temperatures circumvent the photoperiod stimulus leading to a more pronounced increase in cold hardiness and 25 kDa accumulation. According to this study, the water content of plant leaves maintained under natural photoperiod was lower than that of plants grown under extended photoperiod, regardless of the temperature regime. This may

be due to short-day-induced cellular dehydration that, in turn, triggers 25 kDa dehydrin accumulation in *Rhododendron* leaves. The dehydrin of interest is also induced by water stress, while exogenous abscisic acid (ABA) application failed to trigger the accumulation of 25 kDa dehydrin in leaves. Our data show that the 25 kDa dehydrin accumulates preferentially in the leaf lamina, and to a significantly lower levels in the midrib. This differential accumulation is perhaps related to midrib being the primary area of injury in *Rhododendron* leaves.

Conclusions

This study leads to the following major conclusions:

- Several dehydrin species (a total of 11, ranging from 25 to 73 kDa) were found to be up-regulated in 24 *Rhododendron* species during cold acclimation
- The 25 kDa dehydrin appears to be highly conserved in this genus and accumulated with the highest consistency, being present in all the species analyzed with one exception - *R. brookeanum*
 - *R. brookeanum*, a species adapted to tropical regions, is the only species used in the experiments that lacks cold acclimation ability
 - The absolute amount of 25 kDa dehydrin present in a cold acclimated tissues is not an indication of the level of leaf freezing tolerance (LFT)
 - It appears that accumulation of more dehydrins does not necessary translates into more hardiness in different *Rhododendron* species
 - The cold-inducibility (fold increase in the amount of protein) of 25 kDa dehydrin in a given genotype as a result of cold acclimation process appears to be related with its cold acclimation ability (fold increase in the cold hardiness)
 - The 25 kDa dehydrin is not specifically regulated by cold, but is also induced by water stress. No up-regulation was detected as a result of ABA (abscisic acid) treatment

- The 25 kDa dehydrin accumulation in the first stage of cold acclimation is triggered by short day-length in the absence of low temperatures

- Low temperatures exposure during the second stage of cold acclimation causes a more significant increase in 25 kDa accumulation. This accumulation pattern in response to photoperiod and temperature closely mirrors cold hardiness changes during seasonal cold acclimation

- The 25 kDa dehydrin accumulates to higher levels in the leaf lamina compared to the midrib which is also the most sensitive of all leaf tissues to freeze-injury

APPENDIX: DEACCLIMATION STUDY IN *RHODODENDRON*

Introduction

Seasonal fluctuations in cold hardiness (cold acclimation and deacclimation) are believed to result from a combination of physiological and molecular changes that tissues undergo during the annual cycles. In the past, the study of altered gene expression and physiological changes associated with cold acclimation in woody perennials has been the subject of few investigations (Wisniewski and Arora; 2000), but little is known about the changes associated with deacclimation. This process (a decrease of tolerance to low temperature during spring, reaching a minimum in the summer) is equally important because untimely mid-winter thaw followed by hard freeze and/or late spring frosts can cause severe damage to the flower buds and/or ornamental foliage of many horticultural important crops, such as *Rhododendron*.

In the spring, the cold hardiness of trees is progressively lost due to increasing temperatures and longer photoperiod (Dormling 1993; Taulavuori et al. 1996). Also, the loss of dormancy in spring is associated with favorable temperatures and occurs simultaneously with deacclimation, after the chilling requirement of the plant was met (Sakai and Larcher 1987). However, no scientific data exist on the deacclimation kinetics (timing and speed) and the protein changes associated with deacclimation in *Rhododendron*.

The influence of photoperiod on deacclimation has received less attention than the influence of temperature. Taulavuori et al. 1996 reported that photoperiod have little influence on loss of cold acclimation in *Pinus silvestris*. In *Rhododendron*, deacclimation was accelerated when high temperatures and long photoperiods were combined, but the temperature appears to be the most significant factor (Cameron and Dixon 2000). The study of deacclimation in several *Rhododendron* species varying in their cold acclimated hardiness would serve as a ground work for breeders that try to combine deacclimation properties with desirable ornamental traits.

Material and methods

Plant material

For deacclimation experiments, four-five years old containerized cold hardened plants of the following species were used: *R. maximum*, *R. ponticum*, *R. carolinianum*, *R. dichroanthum* and *R. arboreum* representing less, moderate and extremely hardy rhododendrons based on their leaf freezing tolerance (LFT) (Chapter 2).

Deacclimation protocol

Cold acclimated plants were maintained over winter in the field under natural conditions and transferred at the beginning of February to a controlled deacclimation regime. The deacclimation conditions were 22°C and $\sim 150 \mu\text{Em}^{-2}\text{sec}^{-1}$ photoperiod (14/10; D/N) for a period of four weeks. Previous experiments indicated that plants of *R. cv. "Roseum Pink"* exposed to these conditions completely deacclimated in 2-3 weeks (unpublished results). For a time-course study of LFT and protein changes associated with deacclimation, leaves were collected (randomly from 3-4 plants/species) from the previous year's growth every three days starting with day zero (fully cold acclimated samples) over a four week deacclimation period.

Relative cold hardiness estimation

LFT was determined using the procedure of Lim et al. (1998), described in detail in the previous chapters.

Results

LFT of cold acclimated plants (expressed as T_{max}) varied from -46.1°C in *R. maximum* (the most hardy species) to -23.6°C in *R. dichroanthum* (the most tender species). The other three species: *R. carolinianum*, *R. arboreum* and *R. ponticum* had a T_{max} of -41.2°C, -29°C and -36.2°C respectively (Table 1). At the end of 4 weeks deacclimation the differences in hardiness between species were essentially not significant (Table 1), and varied between -7.3°C and -9.8°C. The initial drop (after three days) in LFT was dramatic for all the species, however, this was most prominent (at the highest rate) in *R. maximum* and *R. carolinianum*, two of the most hardy species used in this experiment (Fig. 1). Moreover, the deacclimation kinetics presented in Fig. 1 indicate that *R. maximum* and *R. carolinianum* have overall (over four weeks deacclimation period) the slowest rate of deacclimation whereas *R. dichroanthum* appears to have the fastest rate.

Discussion

Apparently, exposure for only three days to deacclimation conditions leads to a significant reduction of cold hardiness in all species, being more evident in super hardy species such as *R. maximum*. However, it is important to note that the hardiness of these plants after that initial drop was still relatively high, and with the exception of *R. dichroanthum*, the plants wouldn't be affected by subsequent moderate frosts. This observation leads to the conclusion that less hardy *Rhododendron* species are more susceptible to untimely frost events compared to their super hardy relatives. This may have important implications in cultural practices and frost protection strategies.

After the initial drop in hardiness, the deacclimation advanced slowly, but at the end of the fourth week all species were still leaf-hardy between -7.3°C and -9.8°C. These levels of LFT appear to be higher than that expected for fully deacclimated *Rhododendron* leaves (Lim et al. 1998). It is possible that whereas 3-4 weeks of controlled deacclimation conditions may be

enough for plants that were also cold acclimated in growth chambers (as it was the case for "Roseum Pink"), longer than four week time period may be required to fully deacclimate field-acclimated *Rhododendrons*. The LFT of the deacclimated plant species used in this experiment was not significantly different regardless of their hardiness in cold acclimated state. This find supports previous research in *Rhododendron* suggesting that the cold acclimation ability rather than non-acclimated hardiness is a better indicator for their winter survival.

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Table 1. Leaf freezing tolerance (expressed as T_{\max} values \pm standard errors) of five *Rhododendron* species during a controlled deacclimation regime

Species	T_{\max} (-°C)						
	CA	3 days	6 days	9 days	14 days	21 days	28 days
<i>R. maximum</i>	46.1 \pm 1.7	25.1 \pm 0.7	28.1 \pm 0.8	27.1 \pm 0.5	16.4 \pm 0.4	12.6 \pm 0.3	9.8 \pm 0.4
<i>R. carolinianum</i>	41.2 \pm 1.7	25.1 \pm 0.9	19.8 \pm 0.7	17.6 \pm 0.5	15.3 \pm 0.2	9.5 \pm 0.1	7.7 \pm 0.2
<i>R. arboreum</i>	29 \pm 1.8	20.1 \pm 1.1	13.6 \pm 0.5	16.2 \pm 0.3	14.4 \pm 0.4	10.9 \pm 0.4	8.5 \pm 0.3
<i>R. dichroanthum</i>	23.6 \pm 0.8	13 \pm 0.6	11.4 \pm 0.2	11.6 \pm 0.3	11.3 \pm 0.5	8.7 \pm 0.3	7.3 \pm 0.3
<i>R. ponticum</i>	36.2 \pm 0.8	28.8 \pm 0.7	21.8 \pm 0.6	19.6 \pm 0.4	15.3 \pm 0.2	12.6 \pm 0.2	9.6 \pm 0.3

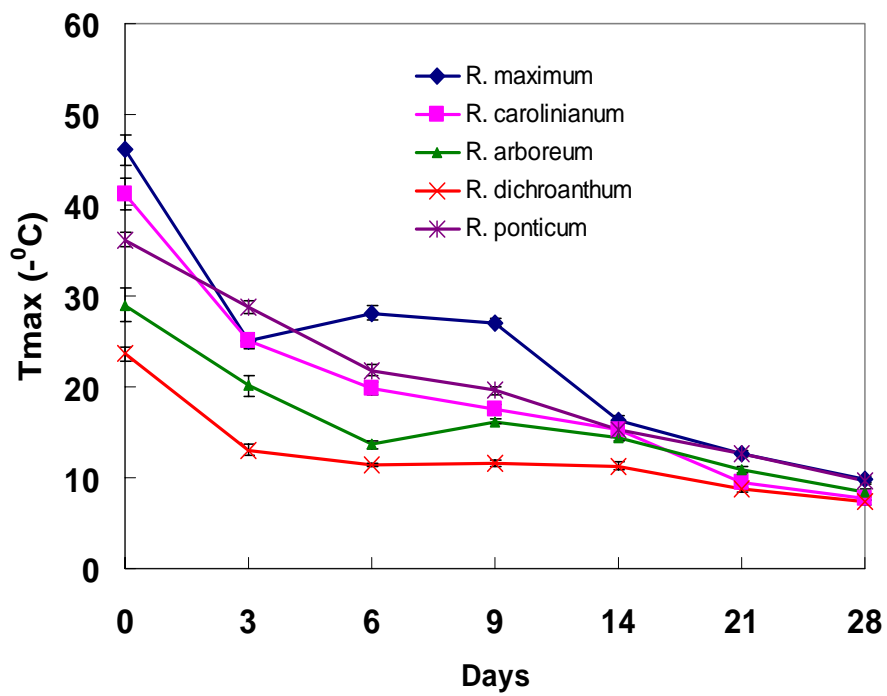


Figure 1. Leaf freezing tolerance (expressed as T_{max}) of five *Rhododendron* species during a four week controlled deacclimation regime