

Environmental Regulation of a 25 kDa Dehydrin in Relation to *Rhododendron* Cold Acclimation

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ABSTRACT. The influence of photoperiod and temperature on the seasonal (fall to winter) cold acclimation and accumulation of a 25 kDa dehydrin in *Rhododendron* ‘Chionoides’ was studied by exposing two groups of plants each in the greenhouse or outdoors to either a natural photoperiod (or short days) or an extended photoperiod (or long days) regime. Results suggest that the shortening daylength alone is sufficient to trigger both the first stage of cold acclimation and concomitant 25 kDa dehydrin induction. Exposure of the plants to natural photoperiod and temperatures induced the greatest cold hardiness and 25 kDa accumulation, while exposure to extended photoperiods (long days) and warmer temperatures (in the greenhouse) failed to induce any significant freezing tolerance in leaves. Whereas short days trigger the cold acclimation process initially, low inductive temperatures can eventually replace the photoperiod stimulus. Seasonal accumulation of 25 kDa dehydrin, on the other hand, appears to be predominantly effected by short photoperiods. Data indicated that the leaf water content of outdoor plants maintained under natural photoperiod was lower than that of plants grown under extended photoperiod. This was also true for the greenhouse plants at the first (September) and the last (January) sampling. It is hypothesized that early 25 kDa dehydrin accumulation may be due to short-day-induced cellular dehydration. Accumulation of two other dehydrins of 26 kDa and 32 kDa molecular masses does not appear to be associated with short day (SD)-induced first stage of cold acclimation. Results show that their accumulation may be regulated by low, subfreezing temperatures and may be associated with the second and/or third stage of cold acclimation of ‘Chionoides’ rhododendron leaves.

Freezing temperatures constitute one of the major environmental constraints limiting growth, development, and distribution of plants. Most tropical and subtropical plant species and herbaceous annuals lack the ability to adapt to cold winters and are typically injured or killed by slightly subfreezing temperatures. In contrast, many temperate woody perennials have the capacity to survive extremely low, winter temperatures (e.g., as low as -269°C in the case of *Cornus sericea* L.; Guy et al., 1986) due to an ability to progressively increase their freezing tolerance (FT) during fall and become maximally cold-hardy by midwinter. This process is called cold acclimation (CA) (Levitt, 1980).

Cold acclimation in woody perennials occurs in at least two stages (Levitt, 1980; Sakai and Larcher, 1987). The first stage (whereby FT increases marginally) is induced by increasingly shorter daylength in early fall whereas the second stage (a more pronounced increase in FT) is induced by low temperatures during late fall and early winter (Fuchigami et al., 1971; Irving and Lanphear, 1967). In woody perennials of the temperate zone, there also appears to be a third stage of CA whereby prolonged subfreezing temperatures induce a further increase in freeze tolerance that culminates in a maximally cold-hardened plant (Weiser, 1970). Cold acclimation is a complex process involving a number of biochemical, physiological, morphological, and molecular changes

(Sakai and Larcher, 1987; Palva and Heino, 1998; Thomashow, 1999; Xin and Browse, 2000). The study of CA physiology of over wintering woody perennials is further complicated by a simultaneous seasonal induction of dormancy making it difficult to separate these two processes (Arora et al., 1992). Species and cultivars within the *Rhododendron* genus that are broad-leaved evergreens offer an opportunity whereby CA physiology can be studied in over-wintering leaf tissues without the interference of endodormancy transitions that occur in other tissues, such as buds, of deciduous woody perennials (Lang, 1987).

Few studies have attempted the investigation of photoperiod and/or low temperature control of CA in *Rhododendron*. A recent, controlled-environment study showed that the combined treatment of short days (8 h) and low temperatures (5°C) induced maximum leaf freezing tolerance (LFT) in *Rhododendron* cv. Hatsugri (Cameron and Dixon, 2000). Väinölä et al. (1999) reported that photoperiod and temperature during the growing season affected the CA ability of *Rhododendron* cultivars when exposed to a controlled hardening regime, in that, the photoperiod played a greater role than low temperature in acclimation of ‘Pohjola’s Daughter’ while ‘Helsinki University’ attained better CA at cooler temperatures and was less sensitive to photoperiod. These studies suggest that both short photoperiod and low temperature are important environmental cues for CA in *Rhododendron*. However, no information exists on the effect of photoperiod and temperature on both the timing and extent of the seasonal development (through the fall and winter) of LFT in *Rhododendron*.

Dehydrins [also known as LEA D-11 proteins (Dure et al.,

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1989)] are hydrophilic proteins known to accumulate in response to cellular dehydration and thought to protect plant cells from it (reviewed by Close, 1996 and Svensson et al., 2002). Leaf tissues of freeze-tolerant species survive freezing stress by tolerating the cellular dehydration that results from extracellular ice formation generating a vapor pressure gradient that causes migration of freezable cellular water to an ice front (Levitt, 1980). Dehydrin proteins and their transcripts have been shown to accumulate during seasonal CA in bark, xylem, buds, shoot apices, and seedlings of a number of woody plant species (Svensson et al., 2002). A definitive *in vivo* role of dehydrins *in planta* has not been demonstrated so far. However, various biochemical properties of dehydrins and their presumed folding characteristics in their native state (Close 1996; Svensson et al., 2002) point to a potential functional role of these proteins in protecting/stabilizing cellular enzyme activities and/or endomembrane structures under dehydration or salt stress conditions.

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 levels of a 25 kDa leaf dehydrin were closely associated with differences in LFT among F₂ segregants, and suggested that this dehydrin could serve as a genetic marker for cold hardiness in this interspecific population. Subsequent studies in our laboratory have shown that the 25 kDa dehydrin is the most conserved (present in 20 of the 21 species) among all the dehydrins detected in a diverse array of *Rhododendron* species, and that it consistently accumulates to high levels in winter-collected leaves of all these species (Marian et al., 2004). However, nothing is known about the environmental regulation (photoperiodic or low temperature) of this dehydrin during the seasonal development of FT in *Rhododendron*.

The objectives of this study were 1) to study the seasonal (fall to winter) development of FT in *Rhododendron* leaves as influenced by natural or extended photoperiods in combination with natural or controlled temperature regimes; and 2) to identify the environmental cue triggering the seasonal accumulation of dehydrins. Additionally, the seasonal leaf water content of these plants was undertaken to explore the hypothesis that one of the physiological links between photoperiod-regulated accumulation of 25 kDa dehydrin and cold acclimation is the reduction in leaf water content.

Materials and methods

PLANT MATERIAL. Five-year-old plants of *Rhododendron* cv. Chionoides, a *R. ponticum* hybrid, were maintained in 20-L pots with artificial mix (1 pine bark : 1 perlite : 1 sphagnum peat) under natural conditions. The plants were periodically fertilized with Azalea Special 21-7-7 (W.R. Grace, Fogelsville, Pa.) at 1.5 g·L⁻¹ plus Fe chelate (Sequestrene 330 Fe, 10% Fe, Ciba-Geigy, Greensboro, N.C.) at 0.25 g·L⁻¹ to maintain pH at 4.5 to 5.5 and electrical conductivity (EC) at 0.5 to 0.9 dS·m⁻² of the potting medium. Plants were watered as needed before being subjected to different treatments in early September.

PHOTOPERIOD AND TEMPERATURE TREATMENTS. In total, 24 plants were used for this study. The experiment was designed to provide four distinct combinations of photoperiod and temperature regimes, and a group of six rhododendron plants was exposed to each of these regimes from September through January. Environmental regimes were 1) natural photoperiod and outdoors (NPOD); 2) extended photoperiod and outdoors (EPOD); 3) natural photoperiod and greenhouse (NPGH); and 4) extended

photoperiod and greenhouse (EPGH). Two groups of six plants each were maintained in the heated greenhouse, the GH group, which were exposed to daily average temperatures of 20 to 24 °C, while the other two groups of six plants each were kept outdoors, the OD groups, under natural fall and winter temperature conditions. One of the two groups of six plants from both the greenhouse and outdoor regimes was maintained under natural photoperiods. The other two groups were supplemented with artificial light ($\approx 10 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at canopy level) provided by incandescent bulbs (Sylvania 75W, GTE Corp. Salem, Mass.) mounted on a wooden frame to provide an EP (long-day) treatment that began in the first week of September and maintained 15 h day/9 h night photoperiod throughout the course of the study. Both the NPGH and EPGH plants were exposed to identical watering regime, i.e. watered whenever needed until the media was saturated. However, outdoor plants (NPOD and EPOD) received only natural precipitation through the course of investigation; no data were recorded for the amount of rainfall/snow during this study. Daily maximum and minimum air temperatures (at the plant height) in the greenhouse and outdoor experimental locations were recorded using a maximum-minimum thermometer. Leaf temperature was monitored by copper-constantan thermocouple (TT-T-30) attached to a thermometer (DP465; Omega Engineering, Stamford, Conn); essentially no differences were observed for NP *versus* EP treatments.

Monthly samples of randomly collected leaves from the current year's growth across all six plants/treatment were obtained from 5 Sept. until 5 Jan. to be used for LFT measurements, protein extraction and dehydrin analysis, and leaf water content measurements. Leaf tissues for dehydrin analysis were ground immediately in liquid nitrogen and stored at -80 °C to be used later.

LEAF WATER CONTENT (LWC). Duplicate samples of leaves from each treatment at each sampling date were weighed fresh, dried at 80 °C for 48 h (or until constant dry weight was achieved), and reweighed. The LWC is expressed as percent dry weight.

RELATIVE COLD-HARDINESS ESTIMATION. Leaf freezing tolerance 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, percent injury estimations, Gompertz functions fitting, determination of T_{max} (temperature causing maximum rate of injury and defined as a measure of LFT) and statistical analysis (multiple *t* tests at *P* = 0.05 to compare mean T_{max} values) were performed as described by Lim et al. (1998).

PROTEIN EXTRACTION, SDS-PAGE, AND IMMUNOBLOTTING. Leaf protein extraction, total protein determination, separation on SDS-PAGE, and immunoblotting with an anti-dehydrin antibody were performed as reported in Lim et al. (1999) with the following modifications. After each acetone wash of the TCA-precipitated protein pellets during the protein extraction procedure, a sterile sealed pipette tip was used to physically break the pellet. This procedure was used specifically to facilitate solubilization of dried pellets and thereby increase protein concentrations in the sample buffer. Dried protein pellets were rehydrated with 100 μL of sample solubilization buffer (Owl Separation Systems, 0.125 M Tris-HCl pH 6.8, 1% (w/v) sodium dodecyl sulfate, 5% (v/v) mercaptoethanol, 15% (w/v) glycerol, 0.005% (w/v) Bromophenol Blue) followed by boiling in a water bath for 5 min. Vigorous vortexing for 30 min was followed by centrifugation at 14,000 g. for 5 min to precipitate the non-protein material.

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

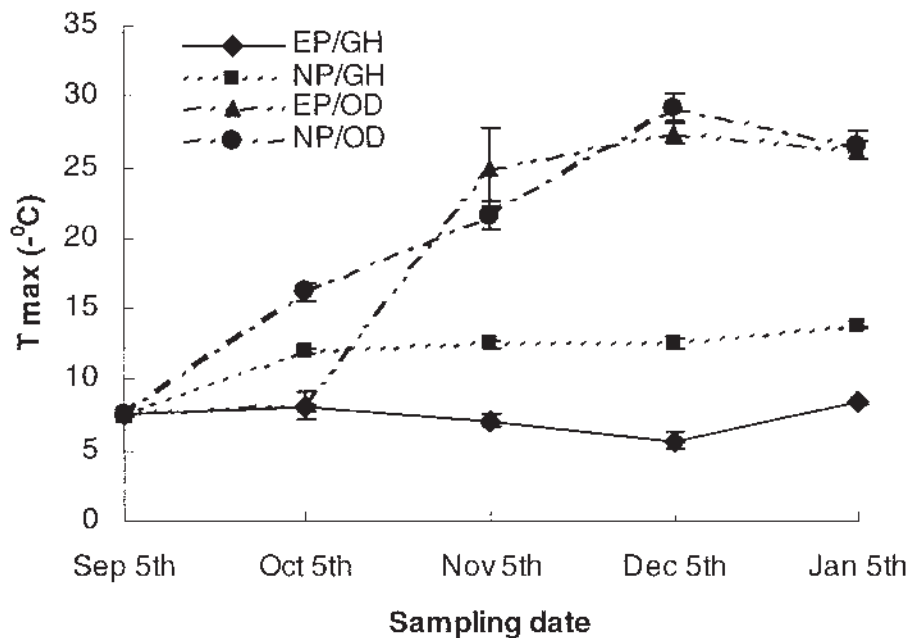


Fig. 1. Effect of various photoperiod and temperature regimes on the seasonal leaf freezing tolerance (expressed as T_{max} , temperature causing maximum rate of injury and estimated from electrolyte leakage assay) of *Rhododendron cv. Chionoides*. EP/GH = extended photoperiod in heated greenhouse; NP/GH = natural photoperiod in heated greenhouse; EP/OD = extended photoperiod outdoors; NP/OD = natural photoperiod outdoors. Error bars are \pm SE of three replications.

Seven micrograms of protein were separated by discontinuous SDS-PAGE. Proteins were electroblotted onto nitrocellulose membranes and anti-dehydrin immunoblotting was carried out according to Lim et al. (1999) using the antibody directed against a synthetic peptide of the highly conserved consensus sequence of dehydrin proteins from several plant species (kindly provided by Timothy Close). Comparison of band intensities among treatments was made visually, i.e., darker bands indicating greater abundance of the protein.

Results

LFT UNDER DIFFERENT PHOTOPERIOD AND TEMPERATURE REGIME. Data indicate that only the treatments that were maintained under NP (in the greenhouse and outdoors) registered an increase in LFT by 5 Oct., 1 month after the first sampling date (Fig. 1). The treatments with supplemental lighting (or EP) in both locations did not show any increase in LFT during this period, regardless of the differences in temperature regimes (Fig. 1). Monthly averages of minimum and maximum temperature were calculated using the daily temperatures recordings at the two locations (Fig. 2).

By 5 Nov., both the NP and EP groups of plants maintained outdoors exhibited an increase in LFT, while neither of these groups of plants maintained in the greenhouse displayed an increase in their LFTs after 5 Oct. (Fig. 1). The LFTs of both of the plant groups maintained outdoors (NP and EP) further increased

through 5 Jan. at which time the LFTs of the NP and EP plants were similar.

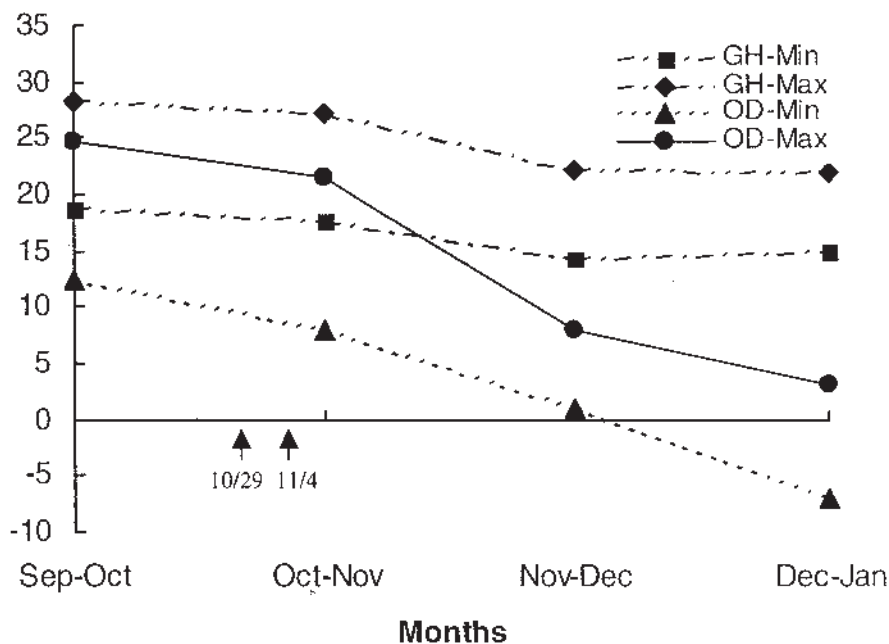
Of the four treatments, the plants exposed to EPGH treatment did not exhibit seasonal cold acclimation, while the plants exposed to outdoor temperatures (both NPOD and EPOD) registered the most pronounced CA (Fig. 1). Plants maintained in the greenhouse under NP (NPGH) acclimated to intermediate levels, but most of this acclimation occurred by the October sampling (Fig. 1).

25 kDa DEHYDRIN ACCUMULATION UNDER DIFFERENT PHOTOPERIOD AND TEMPERATURE REGIMES. The 25 kDa protein was present in all the samples analyzed (Fig. 3A and B). Levels at the start of treatments (September) appeared to be similar to those observed in nonacclimated (NA) summer tissue (July). By 5 Oct., an increase in the accumulation of 25 kDa dehydrin (indicated by darker bands) was already apparent in the plants maintained outside, under natural photoperiod, relative to those exposed to extended photoperiod,

where its level stayed about the same as on 5 Sept. (Fig. 3A). By 5 Oct., the plants maintained in the greenhouse under natural photoperiod also exhibited increased accumulation of 25 kDa, whereas plants exposed to extended photoperiod did not show such accumulation (Fig. 3B).

The 25 kDa dehydrin accumulation began to increase also in the EP-exposed plants by 5 Nov. However, regardless of the temperature regime, its abundance in general was less compared to those exposed to NP throughout the experiment (Fig. 3A and B). Moreover, an accumulation of two other dehydrins of molecular masses of ≈ 26 and 32 kDa, respectively was observed in the

Fig. 2. Minimum and maximum average monthly temperatures for greenhouse and outdoor locations. For example, September to October recording refers to the average maximum or minimum temperatures in the greenhouse (GH) or outdoor (OD) over the sampling interval 5 Sept. to 5 Oct. The first two frosts dates are indicated by arrows.



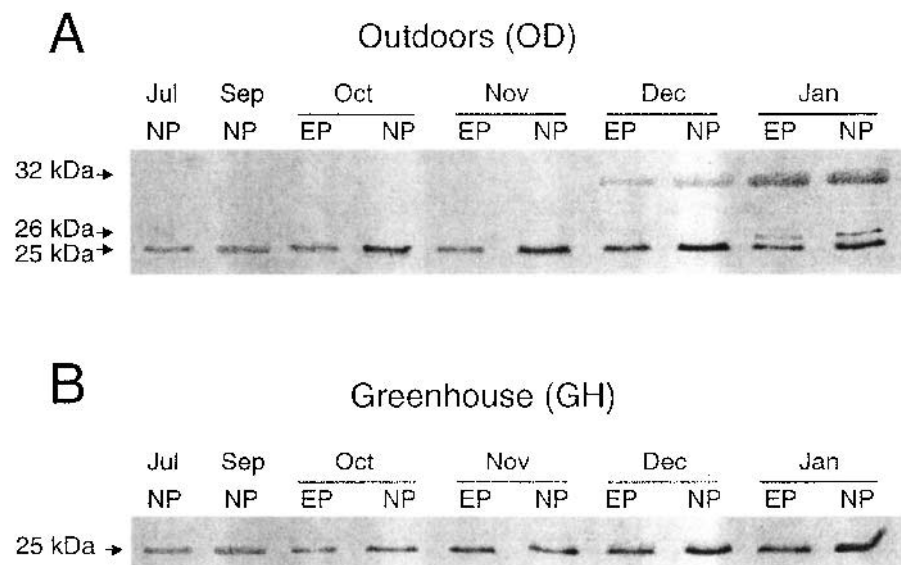


Fig. 3. Effect of various photoperiod and temperature regimes (see methods) on the seasonal accumulation of a 25 kDa dehydrin in *Rhododendron* 'Chionoides' as revealed by antidehydrin immunoblots of leaf proteins (A) Plants maintained outdoors; (B) Plants maintained under heated greenhouse. Seven microgram of total leaf protein was loaded in each lane. NP = natural photoperiod; EP = extended photoperiod

plants maintained outside (natural temperatures) (Fig. 3A); the 26 kDa band was visible only in the leaves collected in January whereas the 32 kDa dehydrin was detected in both December and January samples. These two dehydrins were not detected in the greenhouse-maintained plants (data not shown).

SEASONAL CHANGES IN LWC. Of the two treatments maintained outdoors under natural temperatures, the plants exposed to NP maintained lower LWC (by $\approx 8\%$ to 14%) than those exposed to EP throughout the course of study (Table 1). The differences in LWC between EP and NP plants maintained in the greenhouse were significant only for two sampling dates—September and January—when the NP plants had lower LWC than EP plants. Although, no consistent trend (progressive increase or decrease) of LWC was observed during the course of this study in various treatments, NPOD was the only treatment that showed a significant drop in LWC from September through January at which point it was also the lowest of all treatments.

Discussion

COLD ACCLIMATION. Our results confirmed previous observations by Cameron and Dixon (2000) that low temperatures

and short photoperiods can both induce cold acclimation in rhododendron plants and together they lead to the maximum cold tolerance. Another recent study using controlled conditions demonstrated that a combination of short photoperiod and low temperature had an additive effect on freezing tolerance of *Betula pendula* Roth (Li et al., 2002).

Our results indicate that photoperiod and temperature do not always act in an additive fashion, and that their influence on LFT is seasonally determined. Between September and October the only plants where a significant increase in LFT was observed were those exposed to natural photoperiod indicating that the shortening daylength during this interval—from September (12.75 h) to October (10.5 h)—was inductive for triggering the first stage of cold acclimation (Fig. 1). In the NPGH timeline, further shortening of daylength

from October to January did not result in additional increases in LFT, suggesting that there is a physiological limit to which the *Rhododendron* 'Chionoides' can acclimate in response to short photoperiod alone. It is noteworthy that no significant increase in LFT was noticed in September samples compared to those from July (data not shown). Because the plants for this experiment had starting LFTs comparable to the fully nonacclimated levels in July, we conclude that the effective photoperiod stimulus was a value <12.75 h and >10.5 h. The increase in LFT during the first stage of cold acclimation could be an important adaptation allowing rhododendron leaf tissues to survive initial freezing events of the winter and reaffirms an earlier view (Rinne et al., 1999) that the timing of cold acclimation may be more important for survival of temperate perennials than actual midwinter hardiness.

The gain in NPOD cold-hardiness during the first stage of acclimation (5 Sept. to 5 Oct.), which was about -10 °C, could not be explained in an additive fashion, as the sum of the photoperiod effect (NPGH – EPGH about -5 °C) plus the temperature effect (EPOD – EPGH = 0 °C, Fig. 1). The much higher level of CA in NPOD versus NPGH plants could be due to some interaction between short days (SD) and cooler (nonfreezing) temperature in the OD treatment, which had minimum temperatures ≈ 5 to 10 °C cooler than the GH during the first month. Alternatively, the lower CA capacity of greenhouse plants could be attributed to a higher growth stimulating environment from the somewhat warmer temperatures and regular watering in the greenhouse. This might have caused carbohydrate deficiency in the leaves due to increased night respiration and higher growth activity and, in

Table 1. Seasonal fluctuations in leaf water content (expressed as % of dry weight) of *Rhododendron* 'Chionoides' maintained under natural and extended photoperiods outdoor or in a heated greenhouse (see methods).

Sampling Dates	Water content (% of dry wt)			
	Outdoors		Greenhouse	
	Natural Photoperiod	Extended photoperiod	Natural photoperiod	Extended photoperiod
5 Sept.	124.6 \pm 3.6	134.3 \pm 2.9*	135.8 \pm 2.5	143.4 \pm 3.8*
5 Oct.	132.4 \pm 2.7	150.9 \pm 0.9*	137.9 \pm 2.1	129.9 \pm 5.7
5 Nov.	127.9 \pm 0.4	137.6 \pm 2.1*	141.1 \pm 0.0	141.3 \pm 7.8
5 Dec.	111.3 \pm 0.7	123.2 \pm 0.0*	138.3 \pm 2.3	143.2 \pm 5.9
5 Jan.	114.7 \pm 1.5	130.6 \pm 11.8*	138.1 \pm 5.7	159.5 \pm 4.4*

*Values (means of duplicates \pm SE) significantly different from their natural photoperiod exposed counterparts at $P < 0.05$ (determined by t test).

turn, resulted in lower LFT. Earlier reports, that the development of cold tolerance in *Pseudotsuga menziesii* was favored by low night temperatures (van den Driesshe, 1969) and that the levels of carbohydrates increase in plant cells during cold acclimation (Guy et al., 1992b), support this notion.

A dramatic increase in LFT was observed from 5 Oct. to 5 Nov. in EPOD plants at which point their LFT was statistically similar to that of NPOD plants. Although outdoor monthly average temperatures did not drop significantly during this interval (Fig. 2), two frost episodes were recorded on 29 Oct. and 4 Nov. Cool but above-freezing and subsequent subfreezing temperatures during this sampling period may have induced the second and third stages of cold acclimation resulting in this pronounced increase in LFT of EPOD plants. Comparison of LFT levels of NPOD and EPOD plants at various samplings during September through January suggests that while short days trigger the cold acclimation process initially, low inductive temperatures can eventually 'replace' the photoperiod stimulus. This conclusion is in general agreement with that of Cameron and Dixon (2000), although these authors studied the tolerance-response of *Rhododendron* 'Hatsugiri' to a fixed freeze-stress of -4°C following an acclimation regime of 5 or 20 $^{\circ}\text{C}$ in combination with 8 or 18 h photoperiod rather than seasonal cold acclimation under natural transitions of daylength and temperatures as in our study.

The plants that were not exposed to either SD or low temperatures (EPGH treatment) did not show any increase in cold hardiness. Cameron and Dixon (2000) also observed the greatest freeze damage of all photoperiod x temperature treatments in those plants that were exposed to warm temperature (20 $^{\circ}\text{C}$) and long photoperiod (18 h). These observations suggest that endogenous circadian rhythms do not play a role in seasonal cold acclimation in rhododendrons. Earlier observations that rhododendrons continued active growth in the absence of SD and low temperatures and did not enter into endodormancy (Väinölä and Juntilla, 1998) support this notion. This phenomenon may have practical application in shortening the breeding cycle of this species and thereby hastening physiological maturity by maintaining rhododendrons under extended/continuous photoperiod (Doorenbos, 1955).

PHOTOPERIOD AND LOW-TEMPERATURE INDUCTION OF RHODODENDRON DEHYDRINS. Results from the present study show that shortening daylength during 5 Sept. to 5 Oct. alone is sufficient to trigger the accumulation of a 25 kDa rhododendron dehydrin. This is evidenced by an increased accumulation of this protein in the October samples relative to September collections of the NP-exposed plants in both the greenhouse and outdoors (Fig. 3A and B) coupled with no evidence of such up-regulation in EP-exposed plants from both locations. Among all four treatments, the highest level of the 25 kDa dehydrin (based on visual estimates) was apparent in the NPOD-exposed leaves collected in October to January and may be in response to a combination of progressively shorter photoperiods and lower temperatures from October through January (Fig. 3A). The evidence that low temperatures and short photoperiod together induced maximum accumulation of this dehydrin is further supported by somewhat lower apparent band intensities of this dehydrin in NPGH samples (kept at 20 to 24 $^{\circ}\text{C}$) compared to NPOD plants (Fig. 3A and B). On the other hand, a rather small difference (visually) between the 25 kDa band intensities in December to January samples of NPOD and NPGH plants suggests that, of the two environmental parameters, it was the short photoperiods rather than low temperatures that predominantly effected seasonal accumulation of the 25 kDa dehydrin (Fig. 3A and B). The data also indicate that,

unlike the LFT, photoperiodic regulation of the 25 kDa dehydrin was not replaced by low temperature as shown by substantially higher levels of this dehydrin in the December to January samples of NPOD plants compared to EPOD even though both groups displayed the same LFTs. This explanation is further supported by the dehydrin data for NPGH plants showing that the SD effect on dehydrin accumulation persisted past October, in contrast to its effect on LFT.

Whereas the physiological trigger for low temperature-induced dehydrin accumulation in plant cells is generally believed to be a perceived need by cells to prepare against potential freeze-induced cellular dehydration, the mechanistic basis of short photoperiod-induced accumulation of dehydrins is not well understood. A few studies have suggested a linkage between SD-induced accumulation of certain dehydrins and the reduction of bud water content in *Betula pubescens* Ehrh (Rinne et al., 1998; Welling et al., 1997). More recently, Karlson et al. (2003) have suggested accumulation of a xylem dehydrin in *Cornus sericea* L. to be a prerequisite of photoperiod-controlled reduction of stem water content and prolonged SD exposure. It has also been shown that SD-induced cold acclimation promotes water loss (desiccation) in many woody plant tissues (Chen et al., 1975; Karlson et al., 2003; Li and Weiser, 1971; Li et al., 2002; McKenzie et al., 1974; Rinne et al., 1998) and that artificial dehydration of tissues can increase cold hardiness of many plant tissues including *Rhododendron* leaves (Anisko and Lindstrom 1996 a; 1996 b; Chen et al., 1975; Guy et al., 1992a; Tyler and Stushnoff, 1988).

Therefore we asked the question: is SD-induced up-regulation of 25 kDa dehydrin during the first stage of cold acclimation correlated to reduced LWC? Although the data on LWC did not indicate a consistent seasonal trend, it shows that generally the LWC of NPOD plants was lower than that of EPOD plants (Table 1): whether the reduced LWC is due to cellular dehydration or accumulation of dry matter or both is an open question. Lower LWC in NPGH compared to EPGH plants was observed on two of the five sampling dates. These plants received regular watering which may also explain a relatively higher LWC of NPGH compared to NPOD. In both the locations (GH and OD), NP leaves accumulated relatively higher levels of 25 kDa dehydrin compared to respective EP controls. Taken together, these observations point to a potential association between short photoperiod-regulated LWC and 25 kDa dehydrin accumulation. However, since the seasonal increase in 25 kDa dehydrin accumulation, particularly during the first stage of CA, was not always associated with concomitant lowering of LWC (Fig. 1, Table 1), its SD-induced accumulation cannot unequivocally be explained as a dehydration-induced response.

Increased accumulation of 25 kDa dehydrin was apparent even in the EPGH plants (which did not display any CA) during the course of this investigation, although at substantially lower levels than in NPGH plants. It may, however, be a consequence of altered turnover rate of this protein under extended photoperiod and warmer temperatures. Or it may be in response to an exposure to progressively lower total irradiance over time. Even though the EP plants receive extended daylength, ambient light in the GH decreased in EP as well as NP plants as the season progressed.

The other two dehydrins identified in cold acclimated samples at 26 and 32 kDa, appeared to be induced by low temperatures, not by short photoperiods (Fig. 3A and B). Since these dehydrins accumulated late in December and January after temperatures had dropped below zero (Fig. 2), it is conceivable that their ac-

cumulation, particularly the 26 kDa dehydrin, was specifically triggered by subfreezing temperatures. The relative amount of 25 kDa dehydrin in the maximally cold acclimated leaves of NPGH and NPOD plants does not appear to differ greatly (Fig. 2A and B) but their LFTs do (T_{\max} of -12 and -28 °C, respectively; Fig. 1). This suggests that, at least in 'Chionoides' rhododendron, the 25 kDa dehydrin accumulation alone may be associated with the initial increase in LFT (during the first stage of CA) whereas midwinter hardiness (maximum CA) may result from an additive effect of the 25, 26, and 32 kDa dehydrins.

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