Spring and Fall Cold Hardiness in Wild and Selected Seed Sources of Coastal Douglas-fir

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Abstract

Breeding for increased growth in coastal Douglas-fir (*Pseudotsuga menziesii* (MIRB.) FRANCO) could affect the level of cold hardiness of seedlings used for reforestation. If increased growth is achieved by initiating growth earlier in the spring or prolonging growth later into the fall, cold hardiness could be reduced during these seasons. Cold hardiness was measured in top-cross and first generation seed orchard trees selected for increased growth rates, and wild stand trees throughout one growing season by visual assessment of artificial freeze tests. Significant differences in freezing damage between genetically selected and wild stand trees were found during both the spring and fall. In April, LT_{50} of top-cross trees was 0.7 °C to 2.4 °C below that of wild stand trees, while in October, LT_{50} of wild stand trees was 1.9 °C to 3.4 °C below that of top-cross trees. Mitotic index was investigated as an indicator of dormancy, and a negative correlation between mitotic index and cold hardiness was found. A significant difference in mitotic index between the genetic groups was found once in March when mitotic index in wild stand and seed orchard trees was 1.4 % and mitotic index in top-cross trees was 0.9 %. There were no significant differences in mitotic index at any other times during the year.

Date of bud burst and rates of shoot extension were related to levels of cold hardiness in the three groups of seedlings. The stage of bud burst in May was significantly correlated with levels of hardiness found earlier in March and April. Trees that completed shoot extension earlier in the season were significantly more hardy in the fall. Top-cross trees may extend their growing season later into the fall, thereby gaining a height advantage over wild stand seedlings. These top-cross families do not have an increased mid to late fall frost damage risk, and in fact may have reduced risk of critical spring frost damage due to delayed deacclimation.

Key words: tree improvement, cold hardiness, phenology, mitotic index, artificial freezing.

FDC: 181.22; 181.8; 165.52; 232.12; 422.1; 147.7 *Pseudotsuga menziesii.*

Introduction

Cold hardiness is one of the physiological attributes which determines the environment in which a plant can survive, and conifers are among the most cold tolerant plants (SAKAI and WEISER, 1973). Cold acclimation is triggered by decreasing photoperiod and decreasing temperatures in the fall (WEISER, 1970), while deacclimation in the spring is generally thought to be a result of increasing temperatures, with increasing photoperiod playing a lesser role (VAN DEN DRIESSCHE, 1969; GREER and STANLEY, 1985).

Significant variation in many traits, including hardiness, exists in natural populations of Douglas-fir (MENZIES and HOLDEN, 1981; SKROPPA, 1991; AITKEN et al., 1996; AITKEN and ADAMS, 1996). Genetic improvement programs for this species focus on traits of highest commercial importance including stem volume, form and wood density (WOODS, 1992). Although cold hardiness is of secondary concern for selection, it is important to understand its relationship with commercial selection traits to ensure hardiness is not compromised. Faster growing trees selected for breeding programs must attain a size advantage through one, or a combination of : 1) initiating growth earlier in spring; 2) prolonging the growing season into the autumn; or, 3) growing at a faster rate during the growing season (AITKEN and ADAMS, 1995a). It has been proposed that differences in total growth between trees are caused mostly by variation in the length of the growth period while rate of growth is nearly constant (DORMLING, 1982), suggesting that a longer growing season is the most likely reason for faster growth.

Genetic testing of coastal Douglas-fir since the mid seventies has led to the identification and selection of parents which produce progeny exhibiting superior stem volume growth (HEAMAN and WOODS, 1989). The Douglas-fir trees sampled in this study were planted in 1993 in a trial designed to estimate realized gain in volume per hectare from the British Columbia Ministry of Forests breeding programs. The primary objective of the programs was to develop generalized predictions of unit-area volume gains for a range of genetic levels estimated from individual tree progeny tests (WOODS, 1992).

With reforestation comes the demand for trees which are not only fast growing, but also are well suited for the local climate in which they are planted. The mid-winter cold hardiness usually found in Douglas-fir is sufficient protection under normal winter conditions. Early spring cold hardiness is of great concern in most areas of the Pacific Northwest, however, as this is the period when dormancy is broken and tree growth resumes, and damaging spring frosts are two to three times more frequent than fall frosts (TIMMIS et al., 1994). During periods of active growth, shoot cold hardiness is lowest and newly developing tissue is susceptible to damage. Thus, a thorough understanding of the relationship between levels of cold hardiness and growth phenology in coastal Douglas-fir is required to make wise decisions in reforestation programs.

The objectives of this study were: to compare cold hardiness in Douglas-fir trees produced from wild stand seed, and first-generation seed orchard and top-cross seed selected through breeding programs; to investigate relationships between mitotic index (percentage of dividing cells) in lateral buds and cold hardiness in wild stand and selected trees; and to study date of bud burst and patterns of shoot extension in relation to cold hardiness in wild stand and selected trees.

Material and Methods

Genetic sampling

The three genetic levels investigated in this study originate from the following sources: 1) wild stand seed collected from natural stands, 2) first generation seed orchard seed, and 3) top-cross seed. Collection locations for the wild stand seed and

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for parent trees were distributed throughout the Maritime seed zone south of 51°N (B.C. Ministry of Forests, 1995). Parent trees with phenotypic superiority for stem size, stem straightness and fine branching were selected from wild stands and included in a first generation seed orchard and breeding populations. Random mating among the parent trees under wind-pollinated conditions produced first generation seed orchard seed. Parents of top performing families in progeny tests for stem volume at age 12 were mated together under controlled conditions to produce top-cross seed. Seedling stock from all genetic levels was grown in 615 styroblocks in 1992 using a fully randomized design to control greenhouse environmental effects. Stock was overwintered in the greenhouse, lifted in late February and planted in March of 1993 in a realized gain trial (WOODS, 1992) at a spacing of 3 m by 3 m.

Two test sites in the realized gain trial were monitored for this study. Locations were chosen to represent the breadth of the geographic distribution of Douglas-fir within the seed zone. One site, Holt Creek, is on southern Vancouver Island, (48°45'N, 123°50'W elev. 120 m) near Lake Cowichan. The site has a northeast aspect with moderate slope. The other site is close to the Chehalis River (49°22'N, 121°58'W elev. 390 m) near Mission, British Columbia. This site has a west-southwest aspect and is also moderately sloped. Vegetation and natural regeneration are controlled through manual cutting to ensure trees remain free to grow through to rotation age.

Two blocks on each site were sampled for this study in 1996 and 1997. Each block contains three, 100-tree plots (10 by 10 trees), and each plot contains trees from either the wild stand, seed orchard, or top-cross groups. Allocation of genetic group to plots within blocks was random. Five seedlots from each of the wild stand and seed orchard genetic levels, and five full-sib, top-cross families were sampled in their respective plots. Two trees per seedlot or family per plot were randomly chosen for study. Eight secondary lateral shoot tips from the 1994 whorl were clipped from each of the study trees and placed in individual plastic bags with their identity label. All samples were stored in a refrigerator and underwent artificial freezing within 24 hours. The same trees were sampled on all following dates. The sampling schedule followed in this study is described in *Table 1.*

Freezing methods

Freezing protocols followed AITKEN and ADAMS (1995b). Shoot tips from the 60 trees per site were trimmed to 5 cm and were placed on damp cheesecloth. The cheesecloth was then folded over the shoot tips and packaged in flattened aluminum foil, with open ends to encourage ice nucleation. The packets were hung from a rack in a Forma Scientific Biofreezer (Marietta, Ohio, USA) with air space around all packets, and held for a minimum of five hours at –2°C. Packets containing control shoots were removed at this time. The remaining packets were then cooled at 5°C/hour to each of three test temperatures. Two shoot tips from each study tree were frozen in each of the control and three test temperatures. Samples were maintained at each test temperature for one hour and then packets were removed to a refrigerator at 4°C. The three test temperatures were selected to create a range of damage for

Table 1. – Sampling schedule for the Holt Creek and Chehalis River sites.

Site	Year	Sampling date	Test temp (°C)	Assessment
HOLT CREEK				
	1996			
		Mar. 27	-14,-18,-22	Cold hardiness and mitotic index
		Apr. 25	-6,-10,-14	Cold hardiness and mitotic index
		May 30	-6	Cold hardiness and shoot development
		July 11	-6	Cold hardiness and shoot development
		Aug. 28	-6	Cold hardiness and shoot development
		Oct. 10	-6,-10,-14	Cold hardiness and mitotic index
		Nov. 14	-16,-20,-24	Cold hardiness and mitotic index
	1997			
		Mar. 21	-16,-20,-24	Cold hardiness
		Apr. 10	-10,-14,-18	Cold hardiness
		May 8		Bud burst
		May 20		Bud burst
CHEHALIS RIVER				
	1996			
		Apr. 3	-12,-16,-20	Cold hardiness and mitotic index
		May 8	-8,-12,-16	Cold hardiness and mitotic index
		June 8	-6	Cold hardiness and shoot development
		July 17	-6	Cold hardiness and shoot development
		Sept. 6	-6	Cold hardiness and shoot development
		Oct. 18	-10,-14,-18	Cold hardiness and mitotic index
	1997			
		May 27		Bud burst

2 blocks x 3 genetic levels x 5 seedlots/genetic level x 2 sample trees/seedlot = 60 sample trees per site

needle tissue based on preliminary experiments (–6 °C max, –24 °C min). Temperature control programs were manipulated using a Caltech Scientific model 8000-controller (Richmond, B.C.). After freezing, the packets were placed in the refrigerator overnight and the cheesecloth and samples were removed from the foil the following day. The samples, still in folded cheesecloth, were put into air tight plastic bags and left at room temperature for one week before scoring damage. The needle tissue was then visually scored to the nearest 10% damage.

Mitotic index assessment

Mitotic index describes the percentage of cells in division. The terminal vegetative buds from two secondary lateral branches from each of the 60 trees per site were collected and bud scales were removed. Shoot apices and roughly 5 mm of the subtending shoot was dissected. The fixation and staining protocol followed GROB and OWENS (1993). Apices and the subtending shoot were fixed in 10% neutral formalin for a minimum of 2 hours in a refrigerator. Samples were then washed 3 times in refrigerated distilled water over a 24 hour period to remove all fixative from the bud tissue. Prior to hydrolysis, samples were washed once with distilled water at room temperature. Apices were then hydrolyzed with 5N HCl at room temperature for 50 minutes to produce an adequate balance between hydrolysis and stain receptivity. After hydrolysis, apices were washed once in distilled water, stained with Schiff's reagent, and kept for a minimum of two hours in the dark at room temperature. Specimens were then rinsed three times at 10 minute intervals in sulphur dioxide (SO_2) water followed by washing in refrigerated distilled water. Apices were stored in distilled water in the refrigerator for up to 4 days before squash preparation. The apex was dissected with the tips of two insect pins, placed in a small drop of 45% acetic acid, and squashed into a monolayer using a microscope slide.

Observations on the squashes were done using a Leitz (Wetzlar, Germany) Laborlux S light microscope with 10x ocular and 40x objective lenses. Horizontal scanning was guided with a Leitz 10 mm x 10 mm, square grid ocular micrometer with divisions at 1 mm intervals. Observation of squashes followed the procedure of GROB and OWENS (1993). Cells that contained clearly defined visible condensed chromosomes (clearly prophase-telophase) were counted as dividing. Only one nucleus of an anaphase or telophase combination was counted. Mitotic index was calculated as follows:

Mitotic index = (# dividing cells/ total counted cells) x 100

Shoot development

A visual assessment of the stage of shoot development was used to score degree of bud burst on a scale between 1 and 7 *(Table 2)* on two spring dates at the Holt site and on one date for the Chehalis site *(Table 1)*. The average stage of development was assessed and one bud burst score was given for each of the 60 sample trees.

The length of the leader was monitored for each sample tree at both sites. At each post bud burst sampling, the length of the new shoot was measured from bud scale scar to terminal bud tip. Using the final shoot length, percent of total growth was calculated.

Data analysis

Percent damage due to freezing, percent dividing cells and percent of total growth data were transformed by calculating the arcsin-square root to achieve a normal distribution. Transformed data were analyzed by analysis of variance (PROC ANOVA; SAS, 1988). Hardiness, mitotic index, and percent total growth were compared using the following model:

$$Y_{ijklm} = \mu + B_i + G_j + BxG_{ij} + e_{(ij)k} + s_{(ijk)l}$$

where

μ is the overall mean

B_i is the effect of blocking (i = 1, 2)

G_j is the effect of the genetic level (j = 1, 2, 3)

BxG_{ij} is the effect of the interaction between block and genetic level

$e_{(ij)k}$ is the sampling error

$s_{(ijk)l}$ is the sub sample error

Hardiness between sites or dates was not compared, and only data from test temperatures creating a range of damage among sample trees was included in analyses. Correlations between transformed values of percent damage at the test temperatures creating the greatest range of damage, mitotic index, and percent of total growth were calculated with PROC CORR

Table 2. – Scheme used to estimate the stage of terminal bud burst.

Score	Description
1	A closed, very compact bud (dormant)
2	A moderately swollen, but still entirely enclosed and relatively compact bud
3	A swollen bud that had undergone significant extension, but still had completely intact bud scales
4	A swollen bud, elongated, with only partially (less than 1/3) disturbed bud scales
5	An elongated bud, with most of the bud scales disturbed
6	An elongating shoot completely free of bud scale enclosure
7	A shoot that was completely free of bud scales and significantly elongated

(SAS, 1988) using data from individual sample trees. Linear regression of mean percent damage at the three test temperatures was used to calculate the temperature resulting in 50% damage (LT_{50}) for each genetic level on each sampling date for graphing purposes only.

Results

Tissue freezing

A significant difference in cold hardiness was found among genetic levels on three sampling dates only *(Figure 1).* In early April 1996, top-cross trees at Chehalis River were slightly more frost hardy than seed orchard trees, but cold hardiness of selected trees did not differ significantly from the wild stand trees *(Figure 2a).* In mid-October 1996, small but significant ($P<0.05$) differences in cold hardiness were detected among the genetic levels at both the Holt and Chehalis sites *(Figure 1).* At Holt Creek, wild stand trees were most cold hardy followed by seed orchard and top-cross trees *(Figure 2b).* At Chehalis River, the pattern was similar but only the top-cross and wild stand trees differed significantly in cold hardiness *(Figure 2b).* By mid-November at Holt Creek, cold hardiness was not significantly different among genetic groups *(Figure 1a).* There was no November 1996 sampling at the Chehalis site due to road inaccessibility. On the March and April 1997 sampling dates, cold hardiness was not significantly different among the genetic levels at Holt Creek *(Figure 1a);* however, the ranking of the genetic groups' hardiness was similar to the previous year with wild stand trees displaying either the most or intermediate levels of damage. There was no hardiness testing of the Chehalis site trees due to equipment failure.

Mitotic index

A significant difference in mitotic index among genetic levels was found on the March 1996 sampling for the Holt site only *(Figure 3).* At Holt Creek, the top-cross trees had a significantly lower mitotic index than wild stand or seed orchard trees. There were no significant differences in mitotic index for any other samples and no consistent pattern in ranking of the performance levels by mitotic index *(Figure 3).*

In late March and early April there were significant positive correlations of needle freezing damage with mitotic index for both Holt Creek ($r=0.74$, $p=0.001$) and Chehalis River trees ($r=0.54$, $p=0.005$). One month later, this correlation was significant for Holt Creek trees ($r=0.64$, $p=0.005$) only. In November, there was a significant correlation between needle freezing damage and mitotic index of the newly formed lateral buds for the Holt Creek site ($r=0.63$, $p=0.001$).

Bud burst

Stage of bud burst did not differ significantly among genetic groups in May, 1997 *(Figure 4).* In early to mid-May at Holt Creek, wild stand trees tended to have the most advanced bud development. There were significant correlations between the stage of bud burst in early and late May and needle freezing damage in both March and April for trees at the Holt site *(Table 3).*

Shoot development

There were no significant differences among the genetic groups in percent of total growth for either site over the summer of 1996. At both the Holt and Chehalis sites, there were significant negative correlations between needle freezing damage in mid-October and the percent of total growth that had occurred in June and July *(Table 4).* Lammas growth was observed in August in two seed orchard and two top-cross trees at the Holt site, and one top-cross tree at Chehalis River.

Discussion

In early spring (late March), the Douglas-fir tissues had started to deharden in response to environmental cues. Wild-

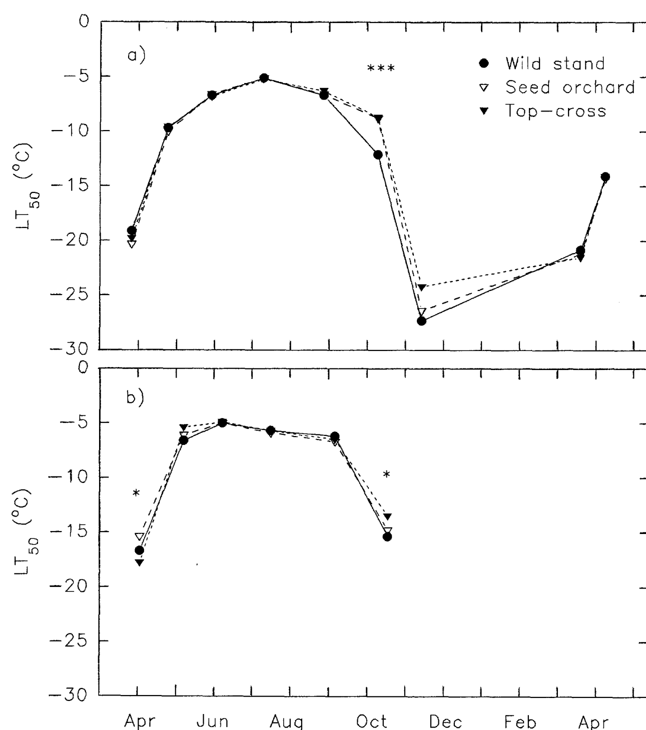

Figure 1. – Cold hardiness (LT_{50} interpolated from percent needle damage at three test temperatures) of trees in three genetic groups in 1996 and 1997 at a) Holt Creek and b) Chehalis River sites. Asterisks indicate a significant difference in percent needle damage among genetic levels as tested by analysis of variance (* = $P \leq 0.05$, *** = $P \leq 0.001$).

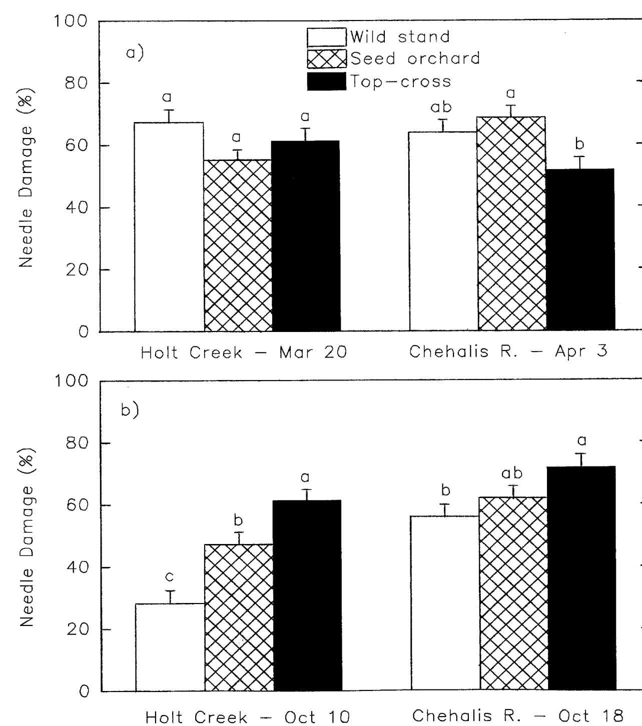

Figure 2. – Mean cold hardiness of trees in three genetic groups in a) early spring and b) early fall 1996 at Holt Creek and Chehalis River sites. Means surmounted by the same letter are not significantly different ($P \leq 0.05$) within each sampling date and site. Error bars indicate standard error of the mean.

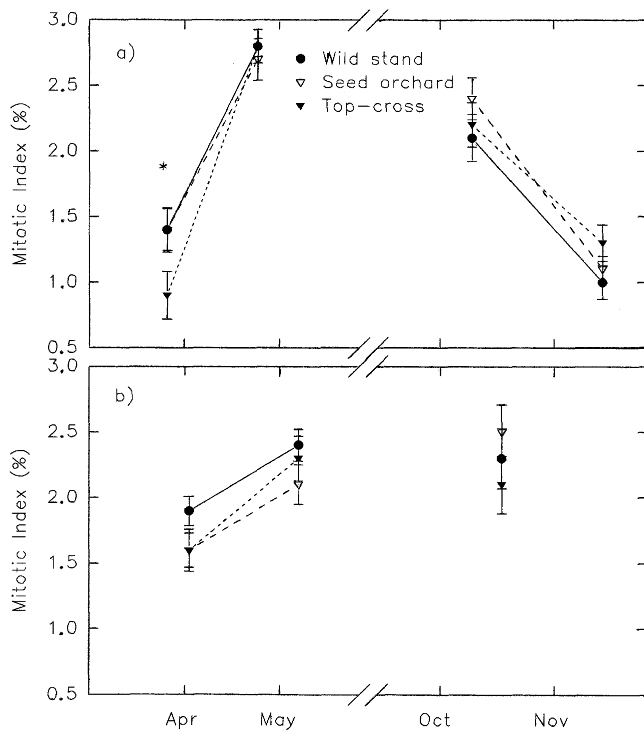

Figure 3. – Mitotic index of terminal buds on lateral shoots in three genetic groups in 1996 at a) Holt Creek and b) Chehalis River sites. Error bars indicate standard error of the mean. Asterisks indicate a significant difference in mitotic index among genetic levels as tested by analysis of variance (* = P ≤ 0.05).

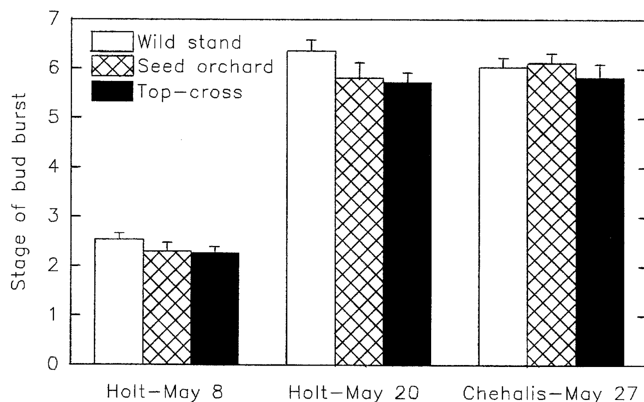

Figure 4. – Stage of bud burst at Holt Creek and Chehalis River sites in May, 1997. Error bars indicate standard error of the mean.

Table 3. – [1])Correlations (r) between bud burst (BB) and cold hardiness (CH) in 1997 for samples from the three genetic levels at Holt (n = 60).

	March CH	April CH	Early May BB	Late May BB
March CH	---	0.5304	0.5892	0.5256
April CH		---	0.6728	0.7552
Early May BB			---	0.8622

[1]) All paired correlations were significant at p ≤ 0.05

Table 4. – [1])Correlations (r) between percent total growth in June and July and cold hardiness (CH) in mid-October 1996 for the Holt and Chehalis sites (n = 60).

	%Growth to June	%Growth to July
CH Holt site	-0.5598	-0.5283
CH Chehalis site	-0.6573	-0.5543

[1]) All paired correlations were significant at p ≤ 0.05

stand trees either showed the most or intermediate levels of damage, while top-cross trees displayed either intermediate or the least amount of damage in artificial freeze tests. Wild stand trees always showed more visible damage than the top-cross group suggesting that the wild stand group had deacclimated to a greater degree by this date. With the transition from dormancy to the initial stages of growth, wild stand trees showed an increased activity in the shoot apex. Top-cross shoot buds were less mitotically active than the wild stand buds and were delayed in their spring development. Significant correlations were found between spring mitotic index and cold hardiness. This supports the concept that the trees which lose dormancy earliest and initiate growth as indicated by increased apical activity are the most susceptible to freezing damage (COLOMBO et al., 1989).

By mid-spring (early May), deacclimation was almost complete in needle tissues, and there was very little variation in cold hardiness among the genetic groups. As bud burst approached, the transition from hardiness to the freezing sensitive growth phase was in progress. During this period, all genetic groups were very similar in their mitotic activity signifying that all trees were at or were nearing complete release from dormancy. The mitotic index was up to twice that of a month prior. Again, a positive correlation was found between mitotic index and cold hardiness.

By examining the progression of bud burst it was possible to gauge the phenological stage of the shoot in terms of its growth initiation. The late spring survey in early May at Holt Creek showed slightly advanced bud development in the wild stand group when compared to the top-cross group. The difference was not large, but was consistent with the trend of top-cross trees being less phenologically advanced at this stage of the year. Previously, timing of bud burst and bud set have been shown to be only weakly correlated with fall cold injury in saplings (AITKEN et al., 1996); however, bud burst and spring cold hardiness are strongly correlated in seedlings (GREG O'NEILL Ph. D., in prep., Oregon State University). In this study, the significant correlations between the stage of bud burst in early May and the needle freezing damages at both spring samplings illustrate the link between an advanced growth phenology and the dehardening process in trees of an intermediate age. These correlations show a direct relationship between the loss of hardiness and the initiation of growth processes. If bud burst was delayed in the top-cross trees, it might allow this group to avoid spring frost damage (DORMLING, 1982). It is possible that late flushing is a result of extended growth into the fall leaving insufficient time for bud maturation before the onset of dormancy. The consequence of this would be a delay in spring bud burst as buds complete maturation. This seems unlikely, however as the genetic groups were effectively equal in hardiness by November.

During the late spring and mid-summer months, it was expected that developing shoots would have little cold hardi-

ness during the period between bud burst and the end of shoot elongation (DORMLING, 1982), and this was indeed the case. In the absence of any substantial hardiness, little variation in needle freezing damage was observed. This was similar to previous studies which indicated that clonal variation in needle cold tolerance was significant on all test occasions except for freezing tests in the summer (NILSSON and WALFREDSSON, 1994).

By monitoring the leader growth throughout the year it was possible to determine the periods when growth occurred and if there were differences among the genetic levels. The shoot elongation rhythm is a reliable measure of early summer phenology (NILSSON and WALFREDSSON, 1994). Needle freezing damage scored in October was negatively correlated with the percentage of total growth that had occurred in late spring and early summer for both Holt Creek and Chehalis River trees. Thus, the trees that had completed more of their growth earlier in the season were more hardy during early fall.

In October, the taller, top-cross trees had the most needle damage in samples from both sites, and the wild stand trees were the least damaged. This would suggest that the top-cross group was least dormant when compared to the seed orchard or wild-stand groups. At Holt Creek, mitotic index was still relatively high in October, and was at similar levels as the late spring sampling indicating that the trees were not dormant. There was no strong relationship between mitotic index and needle damage for either site in early fall.

NILSSON and WALFREDSSON (1994) have stated that negative correlations between height growth and needle cold hardiness in autumn indicate that fall cold acclimation is not easily combined with maximum growth. This appears to be supported by the results of this experiment. Genetic correlation for growth potential and bud set suggest that a network of intercorrelated traits exists within and among populations, and high growth potential is related to delayed bud set (REHFELDT, 1982). This could be extended to propose that trees which complete more of their growth earlier in the year would also have an earlier bud set, and subsequently would be less susceptible to fall freezing damage.

Why would faster growing trees continue to grow into the early fall versus initiating growth earlier in the spring? It has been shown that damaging spring frosts are two to three times more frequent than fall frosts (TIMMIS et al., 1994). If this is the case, then selection against trees which begin growth early in the season would be strong. It would be beneficial to extend the growing season into the least 'dangerous' season of the year. It must be noted, however, that severe freezing events are possible during the late fall period (DUFFIELD, 1956).

For Holt Creek in late fall (November), there was no difference in cold hardiness among the genetic groups and trees also showed a decline in mitotic index as they were in transition to dormancy. Thus, it appears that by late fall all groups possess similar freezing tolerance, and breeding for increased growth has not altered cold hardiness in trees used for reforestation. However, in subsequent generations of 'improved' trees growth phenology and cold hardiness must be monitored to assure that the potential lengthening of the growing season into critical periods is controlled.

Conclusions

Comparisons of the cold hardiness of Douglas-fir trees grown from wild stand seed with those selected in breeding programs for increased stem volume production showed that trees from wild stand seed lost hardiness earlier in the spring, and acclimated earlier in the fall. By late fall genetic groups had similar hardiness levels.

The significant, positive correlation between mitotic index and freezing damage for all study trees suggests mitotic index is an indicator of dormancy and cold hardiness. Wild stand trees lost dormancy and burst bud earlier in the spring, while the top-cross trees entered dormancy later in the fall. Top-cross trees displayed delayed bud burst, which was consistent with a lower early spring mitotic index. This delayed bud burst resulted in greater spring cold hardiness. Shoot extension phenology during the summer was associated with hardiness later in the fall, with early growth completion leading to earlier fall cold hardiness.

Trees selected for faster growth began growth later and continued growing longer. This may result in a decreased risk of spring frost damage, but an increased risk of early fall freezing damage. As damaging spring frosts are two to three times more common than damaging fall frosts (TIMMIS et al., 1994) this selection for rapid stem growth can be expected to reduce overall frost damage risk.

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