AN ABSTRACT OF THE THESIS OF

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Genetics of fall, winter and spring cold hardiness were investigated in seedlings from two western Oregon breeding populations (Coast and Cascade) of coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco). Seedlings of forty open-pollinated families from each population were grown in nursery beds and subjected to either a wet or dry moisture regime. Cold hardiness was assessed in fall and winter after the second growing season, in winter after artificial warming, and in spring prior to budburst in the third growing season, by subjecting detached shoots to artificial freeze tests (AFT), and visually assessing needles, buds and stems for injury. Results of earlier investigations of the same families at age seven were used to compare genetic control of cold hardiness in seedlings and saplings.

Significant ($p < 0.05$) variation in cold hardiness was observed between the two populations in almost all fall assessments and in some cases in the spring assessment. Families within both populations varied significantly in all fall cold hardiness assessments and in the spring assessment. Estimates of individual heritabilities for cold hardiness were weak to moderate, although stronger in spring ($\bar{h}_i^2 = 0.57$) than in fall (Oct $\bar{h}_i^2 = 0.37$), stronger in the wet ($\bar{h}_i^2 = 0.49$) than in the dry regime ($\bar{h}_i^2 = 0.36$), and stronger for stems ($\bar{h}_i^2 = 0.41$) than for needles or buds ($\bar{h}_i^2 = 0.33$). Genetic correlations among the three tissues for cold injury in the same season were generally strong ($\bar{r}_A = 0.69$). Moisture regime had little effect on family ranking for cold hardiness. Fall cold injury in freeze tests was fairly strongly associated with injury from a natural frost that occurred in November of the first growing season ($\bar{r}_A = 0.67$). These results show that cold hardiness of Douglas-fir families can effectively be screened at the seedling stage

using artificial freeze testing. Moderately negative genetic correlations between spring and fall cold injury ($\bar{r}_A$ = -0.47), however, indicate that fall and spring cold hardiness need to be considered separately.

Estimated genetic correlations between seedlings and saplings in stem cold injury were strong in both populations for both spring ($r_B \geq 0.78$) and fall ($r_B \geq 0.80$), indicating that cold hardiness is largely under similar genetic control at the two ages. This means that artificial selection for cold hardiness at either age will be quite effective in improving cold hardiness at the other age as well. Estimated genetic correlations between bud burst date and stem cold injury in the spring was strong in both seedlings ($r_A \leq -0.82$) and saplings ($r_A \leq -0.90$), indicating that bud burst timing is a good predictor of stem cold hardiness prior to bud burst at both ages. Date of bud set, on the other hand, is a reliable predictor of fall cold hardiness only in seedlings ($r_A \geq 0.65$). At the sapling stage, estimated genetic correlations between bud set and fall cold injury to stems were weak ($r_A \geq 0.28$).

Genetic correlations of stem cold hardiness in winter after warming, with cold hardiness in winter prior to warming, and with spring cold hardiness, were strong (r_A = 0.68 and 0.75, respectively) in the Coast, but weak in the Cascade population (r_A = -0.12 and 0.15, respectively). However, estimated heritabilities for cold injury to stem tissue were weak in mid-winter, both before ($\bar{h}_i^2$ = 0.22) and after ($\bar{h}_i^2$ = 0.21) an 11-day warming treatment, compared to in spring ($\bar{h}_i^2$ = 0.68) (calculated for wet regime only). Consequently, response to screening or breeding for mid-winter stem cold hardiness following warming is expected to be weak, particularly in Cascade populations.

Genetics of Fall, Winter and Spring Cold Hardiness in Coastal Douglas-fir Seedlings

by

Gregory A. O'Neill

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DEDICATION

To Charles "Chuckar" Day, who brought biology to life for his students.

Genetics of Fall, Winter and Spring Cold Hardiness in Coastal Douglas-fir Seedlings

Chapter 1
General Introduction

Cold adaptation of planting stock is of concern in the Pacific Northwest where cold injury can reduce growth, stem form quality, and survival of tree seedlings (Timmis *et al.* 1994), saplings (van der Kamp and Worrall 1990), and even mature trees (Duffield 1956). Constraining tree breeding zone sizes addresses this concern by confining deployment of improved genotypes to within their geographic zone of origin, but increases reforestation costs by increasing the number of tree improvement programs required in a region. Also, restricting the area to which improved materials may be deployed limits potential economic gains of tree improvement programs. Therefore, methods to screen individuals and families for cold hardiness would make it possible to identify the most cold hardy genotypes for use on frost prone sites, and thereby broaden breeding zone sizes, and to breed for varieties adapted to cold (Wheeler *et al.* 1990).

Cold adaptation of individuals and families can be assessed after natural frost events in field trials. However, the intensity and frequency of natural frosts may not be uniform across field sites, and may not inflict levels of cold injury capable of discriminating among families, particularly on the typically mild, productive sites where field tests are often established. In addition, the effects of frosts may be confounded with injury due to other causes, such as disease, nutrient deficiency, water-logging or drought. Artificial freeze testing has been proposed as a solution to the deficiencies of field tests in screening for cold hardiness because it can be done uniformly and can provide an objective and inexpensive estimate of cold hardiness (i.e., ability to withstand low temperature without injury) for large numbers of genotypes (Wheeler *et al.* 1990).

In the Pacific Northwest, both seedlings and saplings have been employed in investigations of cold hardiness in coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco) (Campbell and Sorensen 1973; Rehfeldt 1979, 1983a,b, 1986; Schuch *et al.* 1989a,b; van der Kamp and Worrall 1990; Timmis *et al.* 1994; Aitken and Adams 1995a,b, 1996, 1997; Aitken *et al.* 1996; Balduman *et al.* 1999). Seedlings, however, may be at greater risk of cold injury in

plantations (van Haverbeke 1987; Wheeler *et al.* 1990) due to their small size, proximity of their foliage to frost layers on the ground, and their tendency to continue growing into late summer or early fall (Campbell and Sorensen 1973). Thus, it seems crucial to assess cold hardiness at the seedling stage. In addition, seedling tests require less space and provide more uniform test conditions than tests of older trees, resulting in greater statistical precision and cost effectiveness. Also, if ranking of families at the seedling stage is the same as in older trees, early testing of cold hardiness will evaluate cold hardiness of more mature trees as well.

Growing season soil moisture conditions vary greatly throughout the Pacific Northwest and have a strong effect on bud phenology and cold hardiness of Douglas-fir seedlings (White 1987; Joly *et al.* 1989). It is therefore important to determine the degree to which family variation and ranking for cold hardiness within breeding populations is influenced by summer soil moisture conditions. Consequently, a desirable property of cold hardiness testing is that family rankings for cold hardiness remain the same across a range of soil moisture conditions.

Efficiency of cold hardiness testing might be improved through the use of surrogate cold hardiness traits which are either cheaper or easier to assess than cold hardiness itself. A particularly promising predictor of stem cold hardiness is bud phenology. Timing of budburst and cold hardiness of stems in early spring showed a strong, positive genetic relationship in Douglas-fir saplings (Aitken and Adams 1997), although the relationship between bud set timing and fall cold hardiness was weak (Aitken *et al.* 1996). The genetic correlation between timing of bud set and fall cold hardiness, however, may be stronger in seedlings because bud set occurs much closer to the time of fall cold hardening in seedlings than in saplings (Campbell and Sorensen 1973).

While risk of cold injury is considered greatest in spring and fall (Timmis *et al.* 1994), global warming may result in brief periods of unseasonably warm winter weather which could predispose forest trees to premature dehardening (deacclimation) and subject them to damage from subsequent winter frosts (Cannell and Smith 1986; Cannell 1989a; Hänninen 1991). The degree of genetic variation in cold hardiness following a period of unseasonably warm mid-winter weather, and the extent to which such hardiness is under genetic control, determine whether artificial selection and breeding could be effectively utilized to improve cold hardiness under this scenario. In addition, understanding genetic relationships of cold hardiness after mid-

winter warming, with winter or spring cold hardiness, might provide insight into the physiological and genetic control of dehardening, and allow prediction of the potential impacts of selection for winter or spring cold hardiness on cold hardiness following mid-winter warming. For example, a strong genetic correlation between spring cold hardiness and cold hardiness after mid-winter warming, would imply that the two traits are controlled by similar sets of genes and that artificial selection for improved spring cold hardiness would indirectly improve cold hardiness after mid-winter warming.

The issues identified in this introduction raise several questions:

- What is the degree of genetic variation and heritability of cold hardiness in seedlings?
- Are family rankings for seedling cold hardiness traits the same in different moisture conditions?
- To what extent is the genetic control of cold hardiness in seedlings the same as in saplings?
- Are family rankings for cold hardiness traits the same as for phenology traits?
- What is the degree of genetic variation and heritability of cold hardiness after mid-winter warming?
- Is family ranking for cold hardiness after mid-winter warming consistent with ranking for cold hardiness in fall, winter (without warming) and spring?

These questions were addressed in a seedling nursery test where cold hardiness was assessed in fall, winter and spring after the second growing season using artificial freeze testing of cut shoots. This seedling study capitalized upon an earlier investigation of sapling cold hardiness of field-grown trees, by utilizing the same 40 Douglas-fir families from each of two western Oregon breeding populations that were used in the field trial. This allowed corroboration of results and assessment of genetic associations between traits at the two ages. Inclusion of two soil moisture regimes in the nursery test during the second growing season allowed assessment of the influence of summer soil moisture on cold hardiness ranking of families. In addition, a damaging natural frost in the fall following the first growing season in the nursery provided an opportunity to evaluate the degree to which artificial freeze testing predicts susceptibility to natural frost events. Genetic variation in cold hardiness after mid-winter warming was studied by detaching shoots in early February and subjecting one set of shoot samples from all families to artificial freeze testing immediately, and a second set to freeze testing after an 11-day artificial warming period.

Cold injury of families after warming was compared with cold injury immediately prior to warming, and to cold injury in spring when there was no winter warming.

Fall and spring seedling cold hardiness, including the role of soil moisture conditions, are addressed in Chapter 2. This chapter also addresses the degree to which family cold hardiness rankings in artificial freeze tests can be used to predict cold hardiness to natural frost events. Genetic relationships between seedling and sapling cold hardiness, and between cold hardiness and phenology traits at each age, are the subject of Chapter 3. In Chapter 4, the genetics of seedling cold hardiness in winter prior to, and after, warming, are compared to each other and to cold hardiness in fall and spring.

Chapter 2

Quantitative Genetics of Spring and Fall Cold Hardiness in Seedlings from Two Oregon Populations of Coastal Douglas-fir

Abstract

Genetics of fall and spring cold hardiness were investigated in two western Oregon breeding populations (Coast and Cascade mountains) of Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco). Seedlings from forty open-pollinated families from each population were grown in raised nursery beds and subjected to two soil moisture regimes (well-watered and moderate water stress) to evaluate the influence of summer drought on ranking of families for cold hardiness. Artificial freeze testing of detached shoots, followed by visual evaluation of injury, were used to evaluate needle, stem and bud cold hardiness on three dates in the fall (September, October, and November) after the second growing season, and once in the spring (March) prior to the third growing season.

The Cascade population suffered significantly less cold injury than the Coast population in fall tests in almost all cases. However, in spring tests the Cascade population suffered more cold injury than the Coast population, although differences were seldom significant. Families in both breeding zones varied significantly in cold hardiness, with mean estimates of individual heritabilities greater in spring ($\overline{h_i}^2 = 0.57$) than fall (Oct. $\overline{h_i}^2 = 0.37$), in the coast ($\overline{h_i}^2 = 0.39$) than in the Cascade ($\overline{h_i}^2 = 0.32$) population, and in the wet ($\overline{h_i}^2 = 0.49$) than in the dry moisture regime ($\overline{h_i}^2 = 0.36$). Strong estimated genetic correlations were found for cold injury between fall test dates ($\overline{r}_A = 0.80$), indicating that a single test in the fall is adequate for assessing fall cold hardiness. Genetic correlations between spring and fall cold injury, however, were negative and only moderate in magnitude ($\overline{r}_B$ = -0.64 and -0.30, Coast and Cascade, respectively), indicating that cold hardiness needs to be considered separately in the two seasons. Genetic correlations in cold injury between different tissues were moderate-to-strong and positive in both fall ($\overline{r}_B$ = 0.64) and spring ($\overline{r}_B = 0.84$), indicating that artificial selection for increased cold hardiness in one tissue is expected to increase cold hardiness in the other tissues as well. If selection for cold

hardiness is based on only a single tissue, scoring stem damage would result in the greatest genetic gain in cold hardiness over all tissue types. Seedlings grown under summer drought incurred significantly less cold injury in the fall than those that were well-watered; nevertheless, strong genetic correlations in cold injury between moisture regimes ($\bar{r}_B$ = 0.89) indicate that summer moisture conditions had little influence on family rankings for fall cold hardiness. Cold injury after freeze testing in the fall of the second year was fairly strongly associated with both apical bud mortality ($\bar{r}_A$ = 0.67) and needle injury ($\bar{r}_A$ = 0.63) resulting from a natural frost event in November of the first year. Thus, cold hardiness evaluated by artificial freeze testing appears to be a good predictor of cold hardiness during natural fall frost events.

Introduction

Adaptation to cold is among the most important factors limiting crop productivity and quality, and geographic distribution of temperate plant species (Sakai and Larcher 1987; Chen *et al.* 1995). Susceptibility to frost can result in injury, reduced growth, or death of seedlings (van Haverbeke 1987; Timmis *et al.* 1994), saplings (van der Kamp and Worrall 1990; Reich and van der Kamp 1993; Balduman *et al.* 1999) and even mature trees (Duffield 1956). Modeling based on climate records and on empirical data for seedling hardening and dehardening, indicate that Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) in the Pacific Northwest is most susceptible to frost injury in October and the first half of November, and from mid-April to mid-May (Timmis *et al.* 1994). It is well established that cold hardiness varies among geographical sources of Douglas-fir in both the coastal (var. *menziesii*) (Campbell and Sorensen 1973; Larsen 1978; White 1987; Loopstra and Adams 1989; Schuch *et al.* 1989a,b) and interior (var. *glauca*) (Rehfeldt 1979, 1986) varieties, as well as among families within geographical sources (Wheeler *et al.* 1990; White 1987; Aitken and Adams 1995a,b, 1996, 1997; Aitken *et al.* 1996;).

Knowledge of the hardiness of genetic stock to fall and spring frosts is critical to the success of tree improvement programs, for both choosing which seed sources or families to plant in frost-prone sites, and selecting and breeding for improved cold hardiness. In addition, selection for increased growth rate alone may indirectly result in unfavorable changes in cold hardiness (Rehfeldt 1983b; Aitken and Adams 1995b), highlighting further the need for efficient cold

hardiness screening methods and a better understanding of the genetic relationships between cold hardiness and other traits under selection.

Cold adaptation can be assessed by examining cold injury after natural frost events in field trials. There are, however, several limitations to relying on natural frost events for cold hardiness assessment. First, field tests are typically established only for the short term, on mild, productive sites which seldom receive damaging frosts. Second, the effects of infrequent frosts may be confounded with injury due to other causes (e.g., disease, nutrient deficiency, water-logging, drought). Third, the incidence, timing and intensity of frosts are not generally uniform across test sites. Thus, statistical precision for testing cold hardiness differences among families or other genetic entities may be lacking. One solution is to establish supplemental tests on sites particularly susceptible to frost events, but this would entail additional expense, and non-uniform freezing across the site may still be a problem. A better solution is to subject tissue samples to a common test temperature in a freezer (i.e., artificial freeze testing, AFT) and subsequently evaluate the samples for cold injury (Burr *et al.* 1990). In this manner, objective and inexpensive estimates of cold hardiness can be obtained for large numbers of genotypes.

Previous studies by the Pacific Northwest Tree Improvement Research Cooperative (PNWTIRC) on the genetics of cold hardiness in coastal Douglas-fir have employed sapling-aged trees, due to their widespread and immediate availability in established progeny tests (Aitken and Adams 1995a,b, 1996, 1997; Aitken *et al.* 1996; Balduman *et al.* 1999). Seedlings, however, are more prone to cold injury than saplings or older trees (van Haverbeke 1987; Wheeler *et al.* 1990), because of their tendency to continue growing into late summer or early fall (Campbell and Sorensen 1973), their small size, and the proximity of their foliage to frost layers on the ground. In addition, testing for cold hardiness at the seedling stage has advantages over testing at older ages. Seedling tests require less space than field tests and provide more uniform test conditions, resulting in greater statistical precision and cost effectiveness. Also, if ranking of families at the seedling stage is the same as in older trees, early testing of cold hardiness will effectively evaluate cold hardiness at older ages.

If family rankings for cold hardiness are the same at different times in the fall, or at different times in the spring, a single test date would be adequate for assessing family rankings in each season. The earlier study of cold hardiness in saplings showed family rankings were highly

stable across sampling dates in spring prior to bud burst (Aitken and Adams 1997), but were only moderately stable in fall (Aitken and Adams 1996). In the sapling study, spring and fall also differed in the degree to which cold hardiness of different shoot tissues (i.e., needles, stems and buds) were inter-related. While cold injury to these tissues was highly inter-related in the spring, it was less so in the fall (Aitken and Adams 1996, 1997).

The efficiency of seedling tests and ranking of families for different traits can be greatly influenced by the testing environment (Campbell and Sorensen 1978; Kaya 1992). In particular, summer soil moisture conditions have a strong effect on growth phenology and on cold hardiness of Douglas-fir seedlings (Joly *et al.* 1989). Seedlings from southwest Oregon subjected to summer drought set bud earlier and were more frost hardy in early fall than those grown without drought stress (White 1987). It is therefore important to determine the degree to which family variation and ranking for cold hardiness within breeding populations is influenced by summer soil moisture conditions.

The study described in this chapter used artificial freeze testing to evaluate cold hardiness at the seedling stage, in the same two breeding populations and the same forty families within each population, that were investigated earlier by the PNWTIRC in saplings (Aitken and Adams 1995a,b, 1996, 1997). In this chapter the quantitative genetics of fall and spring cold hardiness in two-year-old seedlings is addressed (Objective 1), as well as the influence of summer moisture conditions on family ranking and variation in cold hardiness (Objective 2). In addition, a damaging natural frost in the fall following the first growing season provided an opportunity to evaluate the degree to which artificial freeze testing predicts susceptibility to natural frost events (Objective 3). The relationship between cold hardiness at the seedling and sapling stages is addressed in Chapter 3.

Methods

Materials

The open-pollinated Douglas-fir seed used in this study came from 40 phenotypically selected parent trees (i.e., 40 families) within each of two western Oregon breeding zones (populations) - a Coast breeding zone (US Forest Service Region 6 Breeding Unit 12021) located

in the Siuslaw National Forest in the Coast Range and centered at lat. 44° 20' N, long. 123° 50' W, and a Cascade breeding zone (Bureau of Land Management Breeding Unit 33), located on the lower west slope of the Cascade Mountains and centered at lat. 44° 50' N, long. 122° 30' W. Parent trees in the Coast breeding zone came from an area of approximately 500 km^2, and an elevational range of 67 to 333 m. In the Cascade breeding zone, parents came from a smaller area (approximately 150 km^2), but from a larger elevational range (300 to 833 m). The 40 families within each zone (20 families from each of two 30-family sets) were originally chosen for inclusion in the sapling cold hardiness study (Aitken and Adams 1996, 1997) because seed was available in storage for the present nursery study.

Experimental design

Seedlings were grown in nursery beds in a split-plot design containing four randomized complete blocks. The two main plots (moisture regimes) in each block were subdivided into two replicate sub-blocks (A and B), each containing all 80 families, randomly allocated to sub-plots. Two replicate sub-blocks of the families were needed in each main plot to ensure that adequate numbers of shoots were available for artificial freeze testing. Each family sub-plot consisted of a four-tree row of seedlings. Thus, there was a total of 4 seedlings/sub-plot x 80 sub-plots/sub-block x 2 sub-blocks/main plot x 2 main plots/block x 4 blocks = 5120 test seedlings. Extra seedlings from the test families were used as buffer seedlings, with one buffer row around each whole plot, and three buffer rows between the two sub-blocks in each main plot.

Seedling culture

The two raised nursery beds used in the experiment measured 1.3 x 16 m, were 0.7 m in depth, and were located in Corvallis, Oregon. Beds were divided into 4 m-long main plots, with plastic sheets lining the inside wall of each plot to prevent water movement between plots. Landscape cloth was laid under the beds to prevent seedling roots from penetrating into the ground. The beds were filled with sterilized loamy-sand, and peat was mixed into the top 20 cm at a 1:1 ratio.

Stratified seed were sown at a spacing of 8 cm within rows and 10 cm between rows in early April 1995. Three seed were sown per sowing hole, and thinned at random to a single seedling in the summer. To enhance the temporal and spatial uniformity of mycorrhizal colonization of seedlings, a spore suspension of *Rhizopogon vinicolor* "Trappe 12472" was applied to the

nursery beds weekly for three weeks at 10^7 spores/m^2, beginning one week after sowing. The seedlings were fertilized with 20-20-20 (N-P-K) at a rate of approximately 50 kg/ha (reduced to 25 kg/ha on July 1) every ten days during both growing seasons. The beds were irrigated as required in the first growing season. Symptoms of Lygus bug (*Lygus hesperidus* Hahn) attack (deformed and scarred apical shoots and buds) appeared at the end of the first summer (1995). Lygus injury (presence or absence) was scored on all trees. Visible Lygus bug injury averaged 4% across families.

Two moisture regimes, well-watered (wet) and moderate water stress (dry), were initiated early in the second growing season (mid-June of 1996). A clear plastic canopy was erected over the beds during rainy periods. Soil water potentials were maintained at roughly -0.8 and -1.7 MPa in the wet and dry regimes, respectively, by irrigating to field capacity (wet regime) or with approximately 1.0 cm of water (dry regime) when average pre-dawn soil water potentials of buffer seedlings became more negative than -1.0 (wet regime) or -2.0 MPa (dry regime). Moisture treatments ended on October 18, 1996 (the approximate date of the start of the annual wet season in western Oregon), when plots of both regimes were watered to field capacity and the canopies removed. Seedling survival at the end of the experiment was 98%.

Artificial freeze tests

Because cold hardiness in saplings showed family rankings were more stable across sampling dates in spring than in fall (Aitken and Adams 1996, 1997), this examination of cold hardiness in seedlings focused more on fall than spring cold hardiness, in order to more carefully examine the relationships among cold hardiness at different times in the fall, and thereby identify the optimum fall test date. Previous investigations of sapling cold hardiness also showed family rankings for fall cold hardiness differ considerably among tissues in some cases. Evaluation of cold hardiness in several tissues in the present study was therefore considered necessary, in order to evaluate the need for more than one test tissue, or to identify the optimum test tissue for seedling cold hardiness assessment.

Cold injury from artificial freeze tests (AFTs) was therefore assessed on three occasions after bud set in the second growing season (i.e., in September, October, and November 1996), and once prior to bud burst in the third growing season (i.e., March 1997). On each occasion, the terminal 5 cm of two lateral shoots was harvested from each sampled seedling and subjected to

freezing, one shoot at each of two test temperatures (chosen from preliminary tests - see below), followed by visual evaluation of tissues for cold injury. The possibility that AFT injury scores for needles, stems and buds may differ in their genetic control and variation (Aitken and Adams 1995a, 1996), and in their ability to predict cold hardiness in the field (Simpson 1983), prompted us to examine injury in all three tissues.

Two shoot samples were harvested from each seedling in sub-block 'A' in the September and October tests (begun 20 September and 12 October) and from each seedling in sub-block 'B' in the November and March tests (begun 16 November and 24 March). Second order branch tips were selected from the middle third of each seedling to minimize within-seedling variation in cold hardiness. No attempt was made to avoid second flushed branch tips which constituted about 5% of the samples. On each test occasion, shoots were processed by block, with all seedlings from a single block comprising the sample of a single day. Shoots from the sampled block to be subjected to the warmer of the two test temperatures underwent artificial freeze testing on the day of sampling, while those subjected to the colder temperature underwent testing the following day. Thus, sampling and freezing of all four blocks required eight days in each test period.

Upon harvesting, each shoot was labeled with numbered tape and placed in a plastic bag containing moistened paper towels. Bags were held in an ice chest, then transferred to a refrigerator (2 °C) until packaging for the freeze tests. Groups of approximately 50 shoots were wrapped into flat packets, first in cheesecloth moistened with tap water, and then in aluminum foil. Ends and sides of the packets were pinched closed to minimize desiccation. The packets were placed on a thick aluminum shelf (to facilitate cooling through conduction rather than convection) in a computer-controlled Forma Scientific freezer, model 8270/859M, with a West M3750 temperature controller, and held a minimum of seven hours at -2 °C, in order to freeze extracellular water. The temperature was then lowered to the test temperature at a rate of 3-5 °C/h (Glerum 1985) and maintained at the test temperature for one hour, whereupon the packets were removed from the freezer and placed in a refrigerator (2 °C) to thaw slowly. Once thawed, the packets were transferred to laboratory benches (16-18 °C) where they were held for 6-8 days in order for signs of cold injury to develop in the dark, humid and warm environment inside the aluminum foil packets (Burr *et al.* 1990).

Discrimination among families for cold hardiness is best achieved when family variance in cold injury is greatest, which usually occurs at test temperatures causing an intermediate level of mean injury (i.e., 30-70%) (Aitken and Adams 1996). Consequently, two test temperatures (T1 and T2) were employed in each of the four test periods, to better ensure that the target of 30-70% (and therefore, maximum family variance) mean injury would be obtained.

The two test temperatures for each test date were selected on the basis of preliminary AFT performed the week prior to the experimental AFT. Preliminary AFTs were performed on shoots from buffer seedlings across a range of damaging temperatures, so that temperatures causing an average of 40 and 60% injury could be estimated by interpolation. The three tissues were of similar cold hardiness in all preliminary tests except in November, when buds were considerably more sensitive than needles and stems. Thus, with the exception of November, test temperatures were interpolated from preliminary test injury scores averaged across all three tissues. November test temperatures were obtained from interpolations of preliminary test injury scores averaged over stems and needles only. Temperatures chosen for each test period were -8 and -12 °C in September, -12.5 and -15.5 °C in October, -20 and -22 °C in November, and -15 and -19 °C in March.

Visual evaluation of injury was shown in testing of shoots from field-grown trees to be quite effective in separating families for cold injury (Aitken *et al.* 1996; Aitken and Adams 1996). Seedling shoot samples were inspected through a magnifying lens, with stems cut lengthwise to reveal approximately 2 cm of phloem. Terminal buds were bisected lengthwise to reveal primordial shoot tissues. Needle injury was evidenced by graying, rusting, or abscission of needles, and stem and bud injury by browning or yellowing of normally greenish tissues. Injury to each tissue was recorded as the percentage (to the nearest 10%) of tissue which was damaged. All samples from one block were scored by a single individual to reduce experimental error.

Natural frost injury

Delayed onset of dormancy during the first fall, evidenced by second-flushing of apical or lateral buds of approximately 20% of the seedlings, may have been brought about, in part, by fall fertilization and Lygus bug injury, and may have predisposed seedlings to cold injury from freezes during the early mornings of November 1-5, 1995. Minimum daily temperatures recorded at 1 m above ground level by the Environmental Protection Agency laboratory, located

700 m from the nursery, and within 1 m elevation, averaged -4.5 °C (-3.7 to -5.0 °C) over the five-day period.

Cold injury resulting from the early November frosts made it possible to compare family cold hardiness in a natural frost (NF) to that determined by AFT. Injury from the natural frosts was evidenced by rust colored foliage one week after the frosts, and by aborted apical buds the following spring. The apical shoot and the tips of the upper branches were most severely damaged, with injury grading to least severe at the bottom of the tree. The most severely injured seedlings (11% of total) developed a stunted, 'bushy' appearance following the NF event, which persisted through the third growing season, but none of the seedlings were killed by the frost. NF cold injury was recorded on each seedling, by visually estimating the percent of foliage injured (FI), to the nearest 10%, on November 12, 1995, and by noting apical bud mortality (ABM) (i.e., either dead or alive) in the spring after the frost.

Only non-damaged lateral shoots were used for the AFTs after the second growing season (i.e., 1996). The NF in fall 1995 is not expected to have affected cold hardiness of non-damaged shoots the following year (pers. comm. Les Fuchigami, Oregon State University). Three observations support this opinion. First, all seedlings, including those which were heavily damaged, produced healthy foliage on non-damaged shoots in the spring of 1996. Second, buds which developed on damaged seedlings in the fall of 1996 were large and healthy, and formed within the usual bud set period. Third, seedlings did not appear to sustain any NF injury during the winter of 1996-7.

Statistical analysis

Families were originally grouped and planted by 30-family sets in the field in order to increase the statistical efficiency of comparing large numbers of families. Source population and sets were disregarded when randomizing families to sub-plots in the nursery. Family sets were ignored in the analysis because preliminary results indicated that there were no significant differences between family sets in either zone.

Cold hardiness was evaluated using twelve AFT traits (i.e., cold injury scores of three tissues X four test dates). Preliminary analyses examined family variation in AFT injury at the two test temperatures (T1 and T2) separately, and the mean injury score (T12) averaged across both

temperatures (T1 and T2). In 47 of 48 cases (12 AFT traits X 2 populations X 2 moisture regimes), family F-values based on mean injury scores (T12) were greater than when scores from either of the temperatures were used individually. Consequently, all AFT analyses were based on mean injury scores.

Analyses of the twelve AFT traits were conducted using the following model:

[1] $Y_{ijkl} = \mu + M_k + B_j + MB_{jk} + F_i + FB_{ij} + MF_{ik} + FMB_{ijk} + e_{ijkl}$

where

Y_{ijkl} is the injury score for the l^{th} seedling of the i^{th} family in the j^{th} block in the k^{th} moisture regime,

μ is the overall population mean,

M_k is the fixed effect of the k^{th} moisture regime, with $E(M_k) = M_k$,

B_j is the random effect of the j^{th} block, with $E(B_j) = 0$, and $var(B_j) = \sigma_B^2$,

MB_{jk} is the main plot error, i.e., the random interaction of the k^{th} moisture regime and the j^{th} block, with $E(MB)_{jk} = 0$, and $var(MB_{jk}) = \sigma_{MB}^2$,

F_i is the random effect of the i^{th} family, with $E(F_i) = 0$, and $var(F_i) = \sigma_F^2$,

FB_{ij} is the random interaction of the i^{th} family with the j^{th} block, with $E(FB_{ij}) = 0$, and $var(FB_{ij}) = \sigma_{FB}^2$,

MF_{ik} is the random interaction of the k^{th} moisture regime with the i^{th} family, with $E(MF_{ik}) = 0$, and $var(MF_{ik}) = \sigma_{MF}^2$,

FMB_{ijk} is the random interaction of the i^{th} family with the k^{th} moisture regime and the j^{th} block, with $E(FMB)_{ijk} = 0$, and $var(FMB_{ijk}) = \sigma_{FMB}^2$,

e_{ijkl} is the random within-family plot error of the l^{th} seedling in the i^{th} family in the k^{th} moisture regime in the j^{th} block, with $E(e_{ijkl}) = 0$, and $var(e_{ijkl}) = \sigma_e^2$.

Type III sums of squares of AFT traits were calculated using the SAS GLM procedure (SAS Institute Inc. 1996) and examination of residuals indicated that, with few exceptions, errors were normally distributed and of constant variance. In no case, however, did transformation of the non-normally distributed data materially affect estimates of heritability or family variation of AFT traits. Thus, all results for these traits are based on non-transformed data.

The significance ($p < 0.05$) of the moisture regime-by-family interaction effect was tested with an F-test using the moisture regime-by-family-by-block interaction as the error term. The moisture regime effect was tested using an approximate F-test (Montgomery 1991, p. 262). To compare the efficiency of the two nursery regimes in assessing family differences in cold hardiness, analyses of variance of AFT traits were also performed separately by moisture regime, using a reduced model with all factors containing moisture regime removed from Model [1]. The significance of family variation of AFT traits was tested with the family-by-block interaction as the error term. Each population was treated separately in all statistical analyses, except where the effect of population was examined. In this case the reduced model was employed, but with the addition of factors for population and population-by-block effects, and with families nested within populations. A population effect was tested with an approximate F-test.

Cold hardiness was also evaluated using the two injury traits (FI and ABM) scored in response to the natural frost of November 1995. Because ABM scores were binary (dead or alive), plot mean values were used in all analyses involving ABM. Natural frost injury traits were assessed on all seedlings (both seedling sub-blocks A and B), and before the moisture regimes were initiated. Consequently, there were four replicates of the 80 families within each block. Thus, FI and ABM were analyzed for each population according to a randomized complete block design with 16 replicates of the 40 families. Type III sums of squares for FI and ABM were calculated with the SAS GLM procedure, again using Model [1], but with effects involving moisture removed (i.e., with the reduced model). Because ABM was assessed as a family sub-plot value, the residual error for ABM was the family-by-block interaction. Non-normal distribution of residuals for FI and ABM was corrected by log-transforming values of these traits prior to analysis.

To assess the strength of genetic control of AFT cold injury traits and FI, individual heritabilities were calculated as:

$$[2] \qquad h_i^2 = \frac{3\sigma_F^2}{\sigma_F^2 + \sigma_{FB}^2 + \sigma_e^2}$$

using variance components estimated with the restricted maximum likelihood (REML) estimator in the MIXED procedure of SAS. Individual heritability of ABM could not be estimated because observations of this trait were recorded on plot means. The additive genetic variation (numerator in the heritability equation) was estimated as three times the family variance, rather than four times (as appropriate for half-sib families), because open-pollinated Douglas-fir progeny are expected to be more closely related than half-sibs, due to selfing and mating among relatives (Squillace 1974; Campbell 1979).

Family heritabilities were estimated for FI and ABM to compare the strength of their genetic control, and were calculated as:

$$[3] \qquad h_f^2 = \frac{\sigma_F^2}{\sigma_F^2 + \sigma_{FB}^2/r + \sigma_e^2/ri}$$

where

r = number of replicate blocks for these traits = 16,

i = number of seedlings in each family sub-plot = 4, and

$\sigma_e^2 = 0$ for ABM.

Standard errors of individual and family heritability estimates were estimated according to Dickerson (1969, pages 49-50), using the asymptotic variances of variance components derived in the VARCOMP procedure.

Genetic relationships (i.e., genetic correlations) between AFT cold injury scores in different test periods, between tissues in a single test period, and between natural and artificial frost injury, were evaluated separately by moisture regime. Also evaluated were genetic relationships between the same AFT cold injury trait measured in the dry and wet regime. Type A genetic correlations (r_A) were estimated for traits measured on the same seedlings (i.e., when both traits were measured in sub-block 'A' or both traits measured in sub-block 'B') (Falconer 1986, p. 284):

$$[4] \qquad r_A = \frac{Cov_{F_{x,y}}}{\sqrt{\sigma_{F_x}^2 \times \sigma_{F_y}^2}},$$

where

$Cov_{F_{xy}}$ = estimated family covariance between traits x and y, and

$\sigma^2_{F_x}$ and $\sigma^2_{F_y}$ = estimated family variances of traits x and y, respectively.

$Cov_{F_{xy}}$ is calculated from:

[5] $$Cov_{F_{xy}} = (\sigma^2_{F_{x+y}} - \sigma^2_{F_x} - \sigma^2_{F_y})/2$$

where

$\sigma^2_{F_{x+y}}$ = estimated family variance of $(x + y)$.

Variance components of natural frost injury traits, and the sum of AFT traits and natural frost injury traits (e.g., Nov. needle injury + FI) were also estimated using the REML estimator in SAS MIXED. Type B genetic correlations (r_B) were calculated between traits measured on different seedlings (e.g., trait x measured on seedlings in sub-block 'A', trait y measured on seedlings in sub-block 'B') (Burdon 1977):

[6] $$r_B = \frac{Cov_{\bar{F}_{x,y}}}{\sqrt{\sigma^2_{F_x} \times \sigma^2_{F_y}}}$$

where

$Cov_{\bar{F}_{xy}}$ is the covariance of family mean scores for traits x and y.

Assessment of ABM and FI on both seedling sub-blocks enabled calculation of genetic correlations between NF cold injury and AFT cold injury traits using the same seedling sub-block (i.e., type A genetic correlations - r_A) and different seedling sub-blocks (i.e., type B genetic correlations - r_B). The two genetic correlation estimates (r_A and r_B) for each trait pair were averaged to improve the precision of the estimates.

Results

Genetic variation in AFT scores

The Coast population suffered significantly more ($p < 0.05$) cold injury than the Cascade population in almost all cases in fall AFTs (Table 2.1). In March, however, the Cascade population suffered more cold injury than the Coast population, although differences were statistically significant in only two of six cases. Significant family differences in AFT injury scores were found for all combinations of test date, tissue type, moisture regime and population. The ability to detect genetic variation in cold injury was facilitated by the attainment, in most cases, of intermediate population mean injury scores (i.e., 30-70%).

Mean damage to the three tissues was fairly similar on each test date within the same moisture regime and population, except in November when buds suffered considerably more injury than either needles or stems. In the fall, mean cold injury after AFT was somewhat greater in the Coast (42.3, 60.3 and 49.2% in September, October and November, respectively) than in the Cascade population (34.5, 43.0 and 40.6%, respectively), but in spring, mean injury in the two populations was very similar. Ranges among family means in AFT scores were usually large (Table 2.1). For example, the hardiest family in the Coast population, when grown in the wet regime, suffered only 34% stem injury in October, while the least hardy family suffered 97% injury.

Genetic control of AFT scores

Estimates of individual heritability of AFT scores were generally low to moderate, but varied considerably over seasons and breeding zones (Table 2.1). On average, heritabilities appeared to be greater in spring ($\overline{h_i}^2 = 0.57$ in March) than fall ($\overline{h_i}^2 = 0.25$, 0.37 and 0.21 in September, October and November, respectively), and somewhat greater in the Coast breeding zone ($\overline{h_i}^2 = 0.39$) than in the Cascade breeding zone ($\overline{h_i}^2 = 0.32$). Heritabilities were slightly stronger for stems ($\overline{h_i}^2 = 0.41$) than for needles or buds ($\overline{h_i}^2 = 0.33$ for both tissues). Heritabilities were generally weakest when mean injury scores were less than 30% or greater than 70% (i.e., not intermediate in mean damage).

Table 2.1. Estimated population means, range of family means, family coefficient of variation (CV_F%), individual heritabilities (h_i^2), and significance of population effect (σ_P) for cold injury[a] to needles, stems and buds of seedlings from two Oregon breeding zone populations of Douglas-fir (Coast and Cascade) grown in dry and wet summer moisture environments.

a) Dry

		Coast				Cascade				
Test date[b]	**Tissue**	**Pop. mean**	**Range of fam. means**	**CV_F%**[c]	**h_i^2**[d]	**Pop. mean**	**Range of fam. means**	**CV_F%**[c]	**h_i^2**[d]	**σ_P**[e]
Sept	**Needle**	26.4	10.8 - 42.0	23.1	0.29	18.1	7.3 - 33.7	26.1	0.23	**
	Stem	38.3	14.5 - 60.1	20.9	0.44	29.7	16.7 - 48.8	19.8	0.29	***
	Bud	51.7	27.4 - 69.2	14.3	0.22	41.0	20.5 - 67.1	19.6	0.25	**
Oct	**Needle**	47.3	20.7 - 74.0	20.4	0.31	30.2	11.8 - 54.2	25.8	0.24	***
	Stem	43.8	18.1 - 68.1	28.2	0.53	24.7	6.9 - 47.9	28.4	0.29	***
	Bud	57.3	29.9 - 76.8	16.9	0.33	37.9	14.2 - 63.9	25.6	0.35	***
Nov	**Needle**	16.4	4.4 - 43.4	40.5	0.30	10.7	4.5 - 19.3	17.7[f]	0.06	***
	Stem	22.4	5.9 - 39.2	17.7	0.11	14.4	5.6 - 29.6	25.0	0.22	**
	Bud	86.7	74.3 - 98.5	0.6[f]	0.00	81.1	59.7 - 93.1	5.7	0.10	ns
	Mean	43.4		20.3	0.28	32.0		21.5	0.22	
Mar	**Needle**	61.9	34.4 - 88.6	15.0	0.46	63.2	30.5 - 81.6	15.4	0.50	ns
	Stem	62.7	43.4 - 82.3	12.6	0.47	64.0	47.9 - 84.7	11.7	0.44	ns
	Bud	62.6	46.9 - 86.1	13.0	0.52	63.4	49.7 - 83.7	11.6	0.41	ns
	Mean	62.4		13.5	0.48	63.5		12.9	0.45	

[a] Percent tissue damage after artificial freeze testing of cut shoots.

[b] Three tests (September, October and November) were conducted in the fall of 1996 and one test (March) in 1997.

[c] CV_F% = 100*(square root of family variance)/population mean.

[d] Estimated standard errors of h_i^2 averaged 0.14 (range 0.07 - 0.23).

[e] ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

[f] Family variance component significant at $p < 0.05$; family variance significant at $p < 0.001$ in all other cases.

Table 2.1, Continued.

b) Wet

Test date[b]	Tissue	Coast Pop. mean	Coast Range of fam. means	Coast $CV_F\%$[c]	Coast h_i^2[d]	Cascade Pop. mean	Cascade Range of fam. means	Cascade $CV_F\%$[c]	Cascade h_i^2[d]	σ_P[e]
Sept	**Needle**	35.0	20.5 - 51.7	10.6	0.13	30.8	12.2 - 41.7	16.1	0.23	*
	Stem	45.8	24.3 - 62.2	12.7	0.26	38.7	23.6 - 54.5	11.6	0.15	**
	Bud	56.7	25.7 - 74.8	13.1	0.19	48.4	25.7 - 76.4	20.1	0.31	**
Oct	**Needle**	81.6	66.0 - 96.5	6.6	0.19	71.4	41.7 - 90.4	11.5	0.32	*
	Stem	69.4	33.7 - 97.2	20.3	0.66	50.5	23.3 - 84.0	22.0	0.35	***
	Bud	62.6	27.4 - 88.3	21.5	0.47	43.1	10.8 - 78.2	28.9	0.43	***
Nov	**Needle**	51.7	19.1 - 77.0	24.4	0.49	40.4	14.9 - 70.5	22.5	0.29	*
	Stem	33.3	12.2 - 68.3	31.4	0.45	20.0	9.0 - 38.2	19.6	0.14	**
	Bud	84.8	64.1 - 97.4	6.4	0.17	77.3	47.2 - 92.7	9.7	0.24	*
	Mean	57.9		16.3	0.33	46.7		18.0	0.27	
Mar	**Needle**	58.6	31.9 - 85.1	19.6	0.67	66.2	40.3 - 91.5	14.4	0.52	*
	Stem	58.2	40.4 - 88.1	19.3	0.83	64.5	48.3 - 86.8	13.5	0.55	*
	Bud	60.8	44.1 - 87.4	16.8	0.78	65.1	47.6 - 87.8	13.8	0.70	ns
	Mean	59.2		18.6	0.76	65.2		13.9	0.59	

[a] Percent tissue damage after artificial freeze testing of cut shoots.

[b] Three tests (September, October and November) were conducted in the fall of 1996 and one test (March) in 1997.

[c] $CV_F\%$ = 100*(square root of family variance)/population mean.

[d] Estimated standard errors of h_i^2 averaged 0.14 (range 0.07 - 0.23).

[e] ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

[f] Family variance component significant at $p < 0.05$; family variance significant at $p < 0.001$ in all other cases.

Influence of moisture regime on cold hardiness

As expected, drought accelerated fall hardening; in nearly all cases in the fall months, cold injury was less in seedlings grown in the dry regime (Table 2.1), although significant differences were infrequent (Table 2.2). The only exception to this pattern was bud injury in November, which was very high in both moisture regimes (Table 2.1). The effect of summer drought on cold hardiness dissipated by spring, as differences in cold hardiness between moisture regimes were very small and non-significant in March (Tables 2.1 and 2.2).

In five of six comparisons, heritability estimates in dry regime in September ($\bar{h}_i^2 = 0.29$) were greater than, or equal to, estimates in the wet regime ($\bar{h}_i^2 = 0.21$). However, in October, November, and March, h_i^2 was greater in the wet ($\bar{h}_i^2 = 0.46$) than in the dry regime ($\bar{h}_i^2 = 0.31$) in 16 of 18 comparisons (Table 2.1). Moisture regime appeared to have little or no influence on ranking of families for cold hardiness. In only a few cases was moisture regime-by-family interaction significant, and type B genetic correlations between cold injury scores in the dry and wet regimes were large ($\bar{r}_B = 0.89$; range 0.58 - 1.00) (Table 2.2).

Genetic correlations between tissues

Within a test date, estimated genetic correlations between cold injury scores of different tissue types were consistently strong in March ($\bar{r}_A = 0.84$), but somewhat weaker and more variable in the fall months ($\bar{r}_A = 0.64$) (Table 2.3). Correlations involving stems (i.e., stem-needles and stem-bud correlations) were slightly stronger on average ($\bar{r}_A = 0.75$) than correlations involving needles (i.e., needle-stem and needle-bud correlations) ($\bar{r}_A = 0.68$) or buds (i.e., bud-needle and bud-stem correlations) ($\bar{r}_A = 0.64$).

Genetic correlations between sampling dates

Estimated genetic correlations for injury scores between test dates in the fall were generally strong, averaging 0.80 (Table 2.4). Genetic correlations for injury scores between fall and spring (March) sampling dates were negative, but only moderate in the Coast population ($\bar{r}_B = -0.64$), and weak in the Cascade population ($r_B = -0.30$).

Table 2.2. Significance of moisture regime (dry *versus* wet) (M) and moisture regime-by-family (MxF) effects, and estimated type B genetic correlations (r_B) between moisture regimes for cold injury after artificial freeze testing.

Test date	Tissue	Breeding Zone: Coast M[a]	Coast MxF[a]	Coast r_B	Cascade M[a]	Cascade MxF[a]	Cascade r_B
Sept	Needle			1.00 [b]			0.58
	Stem			0.92			0.79
	Bud			1.00 [b]			0.93
Oct	Needle	*		1.00 [b]	**		0.81
	Stem	**		0.93	**		0.89
	Bud			1.00 [b]			0.81
Nov	Needle	*	*	0.75	**		1.00 [b]
	Stem			1.00 [b]			1.00 [b]
	Bud			1.00 [b]			1.00 [b]
	Mean			0.96			0.87
Mar	Needle		*	0.83		**	0.62
	Stem		*	0.86		*	0.84
	Bud			1.00 [b]			0.87
	Mean			0.89			0.78

[a] Only cases where these effects were significant (* = $p < 0.05$; ** = $p < 0.01$) are noted.

[b] Genetic correlation exceeded 1.00.

Table 2.3. Estimated genetic correlations (r_A) of seedling cold injury scores between needles, stems and buds after artificial freeze testing of seedlings in fall (September, October and November) 1996 and spring (March) 1997.[a]

Test date	Correlation	Breeding Zone	
		Coast	Cascade
Sept	needle-stem	0.99	0.70
	needle-bud	0.75	0.04
	stem-bud	0.75	0.27
Oct	needle-stem	0.97	0.64
	needle-bud	0.54	0.36
	stem-bud	0.78	0.73
Nov	needle-stem	0.63	0.68
	needle-bud	0.88	0.54
	stem-bud	0.65	0.65
Mar	needle-stem	0.76	0.89
	needle-bud	0.62	0.82
	stem-bud	0.95	0.96

[a] Correlations averaged over dry and wet moisture regimes.

Table 2.4. Estimated genetic correlations (r_A) of seedling cold injury scores between test dates in fall (September, October and November) 1996 and spring (March) 1997.[a,b]

Tissue	Correlation	Breeding Zone	
		Coast	Cascade
Needle	**Sept-Oct**	0.97	0.92
	Sept-Nov	0.83	0.87
	Oct-Nov	0.94	0.77
	Nov-Mar	-0.52	-0.39
	Oct-Mar	-0.75	-0.21
	Sept-Mar	-0.76	-0.46
Stem	**Sept-Oct**	0.96	0.87
	Sept-Nov	1.00 [c]	0.85
	Oct-Nov	1.00 [c]	0.98
	Nov-Mar	-0.68	-0.29
	Oct-Mar	-0.47	-0.22
	Sept-Mar	-0.42	-0.21
Bud	**Sept-Oct**	0.90	0.69
	Sept-Nov	0.59	0.52
	Oct-Nov	0.53	0.25
	Nov-Mar	-0.59	-0.34
	Oct-Mar	-0.74	-0.20
	Sept-Mar	-0.78	-0.36

[a] Correlation estimates were averaged over dry and wet moisture regimes.

[b] Genetic correlations between Sept-Oct and between Nov-Mar are type A; all others are type B.

[c] Estimate exceeded 1.00.

Natural frost injury

Although on average only about 3% of the foliage was injured by the natural freeze event in November 1995, the apical bud of approximately 30% of the seedlings was killed (Table 2.5). Nonetheless, genetic control was stronger for FI ($\bar{h}_f^2 = 0.78$) than for ABM ($\bar{h}_f^2 = 0.64$), and family variance, significant for both traits in both populations, was considerably greater for FI (mean $CV_F\% = 52.0$) than for ABM (mean $CV_F\% = 25.9$), although the small population mean value for FI may have upwardly biased its $CV_F\%$ value. Genetic control, however, was stronger for AFT traits ($\bar{h}_i^2 = 0.35$) than for FI ($\bar{h}_i^2 = 0.24$) (Tables 2.1 and 2.5). Genetic correlations between ABM and FI were strong on the Coast ($r_A = 0.76$) and moderate in the Cascades ($r_A = 0.59$). Genetic relationships between FI and AFT traits and between ABM and AFT traits were strong in the Coast population ($\bar{r}_{A\ \text{FI-AFT}} = 0.75$; $\bar{r}_{A\ \text{ABM-AFT}} = 0.81$), but moderate in the Cascade population ($\bar{r}_{A\ \text{FI-AFT}} = 0.50$; $\bar{r}_{A\ \text{ABM-AFT}} = 0.53$).

Discussion

Genetic variation and control

Cold hardiness in conifers tends to be weak in late summer, increases gradually in early fall with the onset of cool nights (i.e., stage 1 of hardening), then increases quickly following the onset of near-freezing nights at the start of the rapid hardening stage (i.e., stage 2 of hardening) (Weiser 1970; Rehfeldt 1979, 1980; Jonsson *et al.* 1981; Kobayashi *et al.* 1983; Nilsson and Wilfridsson 1995), and may reach a maximum upon prolonged exposure to very cold temperatures (Weiser 1970). This model is supported by the observation of a low level of cold hardiness in September (September test temperatures of -8 and -12 °C inflicted intermediate levels of cold injury in most cases), when night temperatures were cool, and a moderate level of cold hardiness in October and November (intermediate levels of cold injury were sustained in most cases in the October and November tests at -12.5 and -15.5 °C, and -20 and -22 °C, respectively), when night temperatures were freezing or near freezing.

Strong genetic control and significant family variation for spring and fall cold hardiness (Table 2.1) corroborate results of studies of cold hardiness in the same families of sapling-age Douglas-fir (Aitken and Adams 1996, 1997; see also Chapter 3 of this thesis), and bodes well for

Table 2.5. Estimated population means, ranges of family means, coefficients of family variation (CV_F%), and individual (h_i^2) and family (h_f^2) heritabilities of two measures of cold injury (FI and ABM)[a] to seedlings from two Oregon populations (Coast and Cascade) of coastal Douglas-fir after a natural frost event in November 1995. Also shown are genetic correlations (r_A) between artificial freeze test traits in the fall and natural frost injury traits.

	FI		ABM	
	Coast	**Cascade**	**Coast**	**Cascade**
population mean % [b]	3.7	2.2	36	21
family range [b]	0.3 - 9.4	0.3 - 9.8	11 - 67	6 - 44
CV_F% [c,d]	49.7	54.3	21.9	29.9
h_i^2 [e]	0.30	0.18	---[f]	---[f]
h_f^2 [g]	0.83	0.73	0.66	0.62
r_A [h]	0.75	0.50	0.81	0.53

[a] FI = foliage injury - visual estimate of the percent of foliage injured; ABM = apical bud mortality - percent of trees in a 4-tree family sub-plot with a frost-killed apical bud.

[b] Based on non-transformed values; all other calculations based on log-transformed values.

[c] CV_F% = 100*(square root of family variance)/population mean.

[d] Family variance significant in all cases at $p < 0.001$.

[e] Estimated standard errors for $h_i^2 = 0.08$ (Coast) and 0.06 (Cascade).

[f] h_i^2 incalculable because ABM values are based on plot means.

[g] Estimated standard errors for $h_f^2 = 0.17$ for both traits in both populations.

[h] Genetic correlations were averaged over wet and dry treatments, over all AFT traits, and over type A and B genetic correlations (see text).

the prospects for genetic improvement of cold hardiness. Greater heritability in October ($\overline{h}_i^2$ = 0.37) than in September ($\overline{h}_i^2$ = 0.25) suggests that hardening is under stronger genetic control at the beginning of stage 2 than in stage 1. Family variation in cold injury (CV_F%) was somewhat greater, on average, in October (mean CV_F% = 21.3) than in September (mean CV_F% = 17.3), perhaps because the more rapid rate of hardening in stage 2 (Weiser 1970) accentuates divergence of families at this stage.

The magnitude of an adaptive response of a population to a cue depends on the probability of adverse conditions in the days or weeks following the cue (Levins 1969). Consequently, greater fall cold hardiness in the Cascade than the Coast population may be a response to more rapidly decreasing minimum daily fall temperatures in the Cascades. Similarly, less spring cold hardiness in the Cascade than the Coast population may be a response to more rapidly increasing minimum daily spring temperatures in the Cascades. The genetic disparity between the Coast and Cascade populations, as evidenced by the difference between the two populations in cold injury, particularly in the fall (Table 2.1), affirms the need for separate breeding zones for Coastal and western Cascade populations. Despite its significance, genetic differentiation between the two populations is exceeded in most cases by genetic variation among families within a population. These results point to the large amount of genetic variation available for natural and artificial selection in coastal Douglas-fir.

Climatic differences between the two sources likely account for the observed differences between the two populations in cold hardiness. However, similar levels of genetic variation within populations (i.e., among families) for the two sources (mean CV_F% = 17.7 for Coast and 18.2 for Cascade) suggest that climatic differences between sources were apparently insufficient to result in different intensities of natural selection in the two populations. Nonetheless, heritabilities were slightly weaker in the Cascade population ($\overline{h}_i^2$ = 0.39 for Coast and 0.32 for Cascade) where winter temperatures are somewhat harsher. A greater range in bud set and bud burst timing among families in the Coast than in the Cascade population may also have contributed to the greater heritabilities of fall and spring cold hardiness in the Coast population (see Table 3.1, Chapter 3). Greater heritabilities for the Coastal than the Cascade population for fall, winter and spring cold hardiness were also observed with saplings of the same families (Aitken and Adams 1996, 1997), and heritabilities of fall cold hardiness of sapling-aged

Douglas-fir families from Washington were greater in a coastal than a Cascade population (Aitken *et al.* 1996).

The three-stage process of hardening (Weiser 1970) is regulated by several factors including chilling (Weiser 1970), soil moisture (Timmis and Tanaka 1976; White 1987), nutrition (Weiser 1970; Alden and Hermann 1971), photoperiod (van den Driessche 1969) and light intensity (van den Driessche 1970), whereas *de*hardening is primarily heat sum dependent after chilling requirements have been met (Campbell and Sugano 1975; Thompson and Moncrieff 1982). The greater number of factors which regulate hardening compared with dehardening, may tend to homogenize mean cold hardiness levels among families in the fall, relative to in the spring, resulting in lower individual heritabilites in the fall. This hypothesis is supported by results for heritability in seedlings ($\overline{h}_i^2$ = 0.28 for fall and 0.57 for spring) and saplings (Aitken and Adams 1996 and 1997), but not by seedling family variation (mean $CV_F\%$ = 19.0 for fall and 14.7 for spring).

Moisture regime

Less cold injury in the dry than the wet regime was observed in 23 of 24 cases (Table 2.1), supporting the contention that drought hastens the onset of hardening. Other drought experiments involving Douglas-fir have also shown greater hardiness in trees from drought-stressed environments (Timmis and Tanaka 1976; White 1987).

Despite differences in cold hardiness in dry and wet regimes, family rankings for cold hardiness were fairly similar in the two moisture treatments, as indicated by the strong genetic correlations between cold injury scores in the two soil moisture regimes and the few cases of significant moisture regime-by-family interaction effect on fall cold hardiness (Table 2.2). Maintaining a moist nursery soil environment is easier than monitoring and maintaining a moderately dry soil environment over the course of a growing season. Spatial uniformity of moisture conditions is also easier to achieve in a moist than a dry soil environment. Furthermore, use of a moist soil environment may facilitate more effective selection for cold hardiness, as average heritabilities were slightly greater in the wet ($\overline{h}_i^2$ = 0.30) than the dry ($\overline{h}_i^2$ = 0.25) regime. Fall phenology was substantially compressed in the dry regime (family range of bud set was 24.6 and 15.6 days in the Coast and Cascade populations, respectively) compared with the wet regime (family range of bud set was 35.4 and 30.1 days in the Coast and Cascade

populations, respectively) (data not shown). Inasmuch as genetic variation in fall cold hardiness is primarily a reflection of family differences in timing of bud set (Aitken and Adams 1996), the wider bud set period in the wet regime may account for the larger fall cold hardiness heritabilities in the wet regime. Strong genetic correlations between the same AFT traits in wet and dry regimes, and greater heritabilities for phenology traits in the wet regime, were also found in four Douglas-fir populations from southwest Oregon (Kaya 1992). These observations suggest that nursery cultural practices will be simpler and selection efficiency slightly greater with the use of a moist soil environment.

The physiological effect of growing season drought on average level of cold hardiness appears to persist for at least a month after the termination of drought. Droughted trees exhibited significantly less cold injury to needles and stems than did non-droughted trees in the November freeze test, one month after the moisture regime was concluded, but the effect disappeared by March, when no differences in cold hardiness between droughted and non-droughted regimes were detectable (Table 2.2). Injury to buds was equivalent in dry and wet regimes in both November and March.

Although summer drought had no effect on mean cold hardiness in the spring following the drought, heritabilities for spring cold hardiness were consistently weaker in the dry ($\bar{h}_i^2 = 0.48$ (Coast) and 0.45 (Cascade)) than in the wet ($\bar{h}_i^2 = 0.76$ (Coast) and 0.59 (Cascade)) regime (Table 2.1). The strong positive genetic correlation between bud set (BS) timing and bud burst (BB) timing (see Chapter 3) would predict that the larger range in BS timing in the wet than in the dry regime would result in a larger range in BB timing in the wet regime, accounting for the larger spring cold hardiness heritabilities in the wet regime. However, BB timing was almost equivalent in the two regimes for both populations. An explanation of the larger heritability of spring cold hardiness in the wet regime is therefore lacking.

An additional factor contributing to the differing heritabilities in the two moisture regimes may involve uneven soil dessication within dry (main) plots during application of the moisture treatment. Uneven dessication resulting in less uniform moisture conditions would contribute to greater variation in cold hardiness within family sub-plots in the dry than in the wet regime. A larger coefficient of variation for error variance for October cold injury scores (i.e., at the cessation of the drought treatment) in the dry (mean CV% = 65.6) than in the wet (mean CV% =

43.2) plots supports the contention that drying may have been less uniform in the dry than in the wet treatment.

Natural frost injury

Genetic correlations between natural frost injury scores (FI and ABM) and fall AFT injury scores (Table 2.5) were moderate to strong, despite differences in year of assessment (NF in 1995, *versus* AFTs in 1996), mean minimum daily fall temperatures (2.8 °C warmer in September 1995 than in September 1996), age of plants (one-year-old seedlings (NF) *versus* two-year-old-seedlings (AFT)), tissues scored (whole seedlings (NF) *versus* detached shoot tips (AFT)), scoring technique (percent foliage injured and proportion of apical buds killed (NF) *versus* percent needle, stem and bud injury (AFT)), and use of different personnel for scoring damage. The strength of these correlations attest to not only the consistency of family rankings for cold hardiness in natural and artificial environments, but also to the effectiveness of the AFT evaluation technique as a prediction tool for relative fall cold hardiness of families. Strong genetic correlations were also observed between Douglas-fir sapling injury due to a natural frost in early May 1992 and sapling AFT injury the following spring (Aitken and Adams 1997). Additional evidence of the ability of the AFT technique to reliably predict cold hardiness ranking in NF events was provided by Rehfeldt (1986), who used scores from natural fall frost injury in interior Douglas-fir to validate a cold injury model developed mainly from artificial freeze tests. Cold hardiness of seedlings in AFTs was also closely related to long-term survival of Scots pine (*Pinus sylvestris*) in a harsh climate in Sweden, where survival was determined mainly by differences in fall frost hardiness (Nilsson and Walfridsson 1995).

Regardless of the strength of the correlation between injury in natural and artificial freeze tests, relying upon natural frost events in common garden tests to assess family differences in cold hardiness is problematic, as frosts which inflict levels of injury adequate for detecting family differences are often infrequent and non-uniform across sites (Campbell and Sorensen 1973; Wheeler *et al.* 1990). Furthermore, the greater individual heritability of AFT injury scores *versus* FI cold injury scores emphasizes the greater selection efficiency associated with artificial freeze testing *versus* the use of NF injury in seedling cold hardiness evaluation. If a NF event did provide the opportunity to evaluate cold hardiness, slightly greater heritabilities for FI than for ABM, suggest that gain in cold hardiness due to artificial selection may be greater for FI.

Nonetheless, apical bud mortality (ABM) is likely more important than foliage injury, because long term plantation value is probably more strongly associated with ABM.

The relatively large amount of injury sustained by the seedlings (e.g., apical buds killed on 30% of the seedlings, and 11% of seedlings developed a stunted, 'bushy' form, which persisted through the third growing season) at -5 °C in the November natural frost stands in contrast to the relatively modest mean cold injury of 22% to stems in the much colder -21 °C (mean test temperature) AFTs in November (although buds were more heavily damaged). This difference (i.e., between injury levels following a natural frost and those observed in AFT) highlights the relative nature of cold hardiness assessment which AFT provides, and emphasizes the risk associated with inferring absolute levels of field cold hardiness from AFTs. Nonetheless, family rankings were fairly similar following the natural and artificial frosts, despite large differences in absolute level of injury resulting from the two frosts.

AFT tissue choice

Moderate to strong, averaged genetic correlations, between tissues in the same fall month or in March, indicate that a single tissue may be adequate for fall or spring cold hardiness evaluation in AFTs. Choice of the optimum tissue for use in AFT cold hardiness evaluation tests should focus on the correspondence between cold hardiness in AFTs and economic impact of frost injury to different tissues in the field, but should also consider ease and reproducibility of measurement, and anticipated gain. While frost injury in AFTs may be easiest to evaluate for needles, occasional injury to needles is not expected to significantly impact survival, growth rate or stem form, and therefore, should not have a major impact on plantation value. Stem injury, particularly near the base of a seedling, is more likely to be lethal than needle or bud injury, but lethal frosts are infrequent in the Pacific Northwest. On the other hand, heritabilities were slightly stronger for stems than for needles or buds, and genetic correlations of AFT traits with natural frost injury traits (FI and ABM) were slightly stronger for stems than for buds and needles, so expected genetic gain in field cold hardiness would be greatest for stems. Furthermore, tissue-tissue genetic correlations involving stems were slightly stronger than tissue-tissue genetic correlations involving buds or needles. Consequently, if AFTs were performed on one tissue only, gain in cold hardiness combined over all tissues would be greatest if stems were used for assessment.

In economic terms, however, cold hardiness of buds may be more important than cold hardiness of stems or needles. Bud injury can cause substantial stem deformation and growth retardation (as observed in this experiment), which has cumulative negative impacts on plantation value (van der Kamp and Worrall 1990; Reich and van der Kamp 1993). Cold injury to buds is commonly reported (van der Kamp and Worrall 1990; Aitken and Adams 1997; Hänninen 1991), and buds are considerably more susceptible to cold injury in late fall than are stems or needles in seedlings (Burr *et al.* 1990; Guak *et al.* 1998; and the present study - Table 2.1) and in saplings, possibly because they may employ a different mechanism to prevent frost injury than do needles or stems (Sakai 1983; Aitken and Adams 1996). In a study of frost injury in frost prone microsites in Douglas-fir plantations in interior British Columbia, it was found that "...trees that escaped [early spring] frost damage by virtue of the fact that their buds were still enclosed in bud scales, showed little progressive dieback" (Reich and van der Kamp 1993). These observations regarding the importance of bud cold hardiness in the field provide further suggestion that stem cold hardiness may be the most important tissue for use in AFT, because gain in stem cold hardiness in AFT will provide the greatest gain in cold hardiness of the apical bud in the field. However, regardless of the tissue selected for cold hardiness assessment, strong variation in the degree of injury at different locations within the seedlings following the natural frost (injury was considerably more prevalent at the shoot apex and branch tips than at the shoot base and near the stem) points to the need to standardize sampling position in cold hardiness evaluation.

AFT test date choice

The weak-to-moderate negative genetic correlations between fall and spring cold injury (FCI and SCI, respectively) indicate that both spring and fall cold hardiness must be considered in an improvement program, and that genetic gain in cold hardiness in one season will partially offset gain in the other. Coast families, with an average (across all tissues, fall months and both moisture regimes) genetic correlation between fall and spring cold injury (FCI-SCI) of -0.64, may be more prone to such antagonism than Cascade families, with an average FCI-SCI genetic correlation of only -0.30. (Family mean phenotypic correlations between fall and spring cold hardiness are illustrated in Chapter 3 in a scatterplot (Fig. 3.1) of October *versus* March AFT cold injury scores.) These results are corroborated with similar FCI-SCI correlations for the same families from both populations at the sapling stage ($\bar{r}_A$ = -0.53 (Coast) and -0.17 ≤ $\bar{r}_A$ ≤ 0.27 (Cascade)) (Aitken and Adams 1995a).

More rapid change in minimum daily temperature in both fall and spring in the Cascade Mountains than in the Coast Range may be expected to result in smaller ranges in family means for BB and BS date, and consequently, in weaker family variation for FCI and SCI in the Cascade population, which in turn, would result in weaker FCI-SCI genetic correlations in the Cascade population. These expectations were realized for BS date (range = 35.4 - Coast and 30.1 - Cascade), BB date (range = 19.3 - Coast and 15.3 - Cascade), and SCI ($CV_F\%$ = 18.6 - Coast, and 13.9 - Cascade), but not for FCI ($CV_F\%$ = 16.3 - Coast, and 18.0 - Cascade) ($CV_F\%$ values averaged over all cold injury traits in wet regime - Table 2.1; BB and BS data not shown).

Genetic correlations for cold hardiness between fall test dates for the same tissue were generally strong. The only exception involved September-November and October-November correlations for buds, as buds suffered severe injury in AFTs in November. Therefore, use of a single fall test date should be adequate for ranking seedling families for fall cold hardiness. Choice of the optimum test date for use in fall cold hardiness evaluation should be a function of frost injury risk at different times in the fall, and expected genetic gain in cold hardiness from selections based on AFTs conducted on different fall dates. October is recommended as the optimum AFT test date for seedlings and saplings because heritabilities for cold hardiness were greatest in this test period, and the peak fall frost injury risk period in the Pacific Northwest includes October (Timmis *et al.* 1994).

Chapter 3

Genetic Relationships between Seedling and Sapling Cold Hardiness of Coastal Douglas-fir and Implications for Selection

Abstract

Genetic control of cold hardiness in two-year-old seedlings was compared to that in seven-year-old saplings of 40 open-pollinated families in each of two breeding populations (Coast and Cascade) of coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco) from western Oregon. In addition, the potential of using bud phenology traits to predict cold hardiness at the two stages was explored.

Fall and spring cold hardiness were assessed using artificial freeze testing (AFT). Similar genetic control of cold hardiness in seedlings and saplings is suggested by strong genetic correlations between seedlings and saplings for fall and spring cold injury traits (r_B never < 0.78), and by similar trends in individual tree heritability estimates (h_i^2) at both ages: e.g., h_i^2 was greater in spring ($\overline{h}_i^2 = 0.73$) than in fall ($\overline{h}_i^2 = 0.36$), and greater in the Coast population ($\overline{h}_i^2 = 0.69$) than in the Cascade population ($\overline{h}_i^2 = 0.40$) at both ages. Strong responses to direct selection are expected for spring cold hardiness at both ages and for fall cold hardiness in seedlings, even under mild selection intensities. Similar family heritabilities in seedlings and saplings, and strong genetic correlations between ages for cold hardiness traits, ensure that selection at one age will produce gains at the other age, which approach those expected from direct selection at the other age. Genetic correlations (r_A) between fall and spring cold hardiness were near zero in the Cascade population ($r_A = 0.08$ and -0.14, ages 2 and 7, respectively) but were moderate and negative in the Coast population ($r_A = -0.54$ and -0.36). Thus, artificial selection for cold hardiness in fall or spring, would have little or no influence on cold hardiness in the other season in the Cascade population, but would have a detrimental impact in the Coast population. Therefore, both spring and fall cold hardiness should be selected simultaneously in Coast populations to ensure that cold hardiness is not reduced in either season.

Bud burst timing appears to be a suitable surrogate to AFT for assessing spring cold hardiness in both seedlings and saplings; expected improvement in spring cold hardiness through selection for late bud burst approaches gains expected when spring cold hardiness is selected directly using AFT. Bud set timing is an effective surrogate to AFT for assessing fall cold hardiness in seedlings, but is a poor predictor of fall cold hardiness in saplings.

Introduction

As coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco) breeding programs advance into the second generation in the Pacific Northwest, there is increasing interest in enlarging the size of breeding zones, and in capitalizing upon the most elite genotypes. The prospect of deploying improved genotypes over larger geographical areas, or of developing varieties adapted to particular stress environments, requires methods to evaluate genotypes for traits related to adaptation (Wheeler *et al.* 1990). Cold hardiness is an adaptive trait of considerable importance in Douglas-fir, as spring and fall frosts can cause extensive damage to planted seedlings (Timmis *et al.* 1994), saplings (van der Kamp and Worrall 1990; Aitken and Adams 1997) and even mature stands (Duffield 1956).

Cold adaptation can be inferred from frost damage observed in genetic field trials. While field tests are useful for assessing growth capacity of populations and families, they have several limitations with regard to evaluating cold hardiness. Field tests are typically established for a short term, on mild, productive sites, which seldom receive damaging frosts. When frosts do occur, damage caused by the frosts may be confounded with the effects of injury due to other causes (e.g., disease, insects, nutritional deficiency and drought). In addition, the incidence, timing and severity of natural frost events are usually not uniform across field sites. Furthermore, if field sites are not visited regularly, damage may go undetected, or the timing of cold injury events may be unknown. A logical alternative to field testing is to collect shoot cuttings from trees in genetic tests in nurseries or field sites, subject the samples to a common freezing temperature, and then visually evaluate shoots for cold injury (i.e., artificial freeze testing - AFT). In this manner, objective and inexpensive estimates of cold hardiness may be obtained for large numbers of genotypes (Aitken and Adams 1995a, 1996, 1997).

A previous study by the Pacific Northwest Tree Improvement Research Cooperative (PNWTIRC) on the genetics of fall and spring cold hardiness in coastal Douglas-fir employed sapling-age materials due to their wide and immediate availability in established progeny tests (Aitken and Adams 1995a,b; Aitken and Adams 1996, 1997; Aitken *et al.* 1996; Balduman *et al.* 1999). Cold hardiness at the seedling stage, however, may be more important than at older stages. Because of their small size, closer proximity to ground level where temperatures are coldest (Landsberg 1986), and their tendency to continue growing, through free or lammas growth, into late summer or early fall (Campbell and Sorensen 1973; Rehfeldt 1983b; Li and Adams 1993), seedlings are more vulnerable to frost injury than saplings or older trees (van Haverbeke 1987; Wheeler *et al.* 1990).

Due to the relatively small area of land needed, the uniformity of nursery environments, and the relatively short duration of nursery evaluations, AFT at the seedling stage may provide cheaper, earlier and more precise estimates of cold hardiness than field trials. Testing at the seedling stage would be particularly valuable if genes controlling cold hardiness of seedlings and saplings were largely shared, such that evaluation at the seedling stage could be used to effectively rank families or genotypes for cold hardiness in older trees.

Chapter 2 of this dissertation described fall and spring cold hardiness in seedlings of the same families (40 from each of two western Oregon breeding zones) investigated at the sapling stage in the earlier PNWTIRC study. The main objectives of this chapter are to compare genetic control of cold hardiness traits at the two stages (seedling and sapling) (Objective 1), and to evaluate breeding implications, including the potential for early testing of these traits (Objective 2).

Strong genetic relationships between timing of shoot growth and cold hardiness would allow bud phenology to be used as a surrogate to AFT for assessing cold hardiness. In saplings, the genetic association between spring cold hardiness and bud burst timing was strong, but the relationship between fall cold hardiness and timing of bud set was weak (Aitken and Adams 1995a, 1997; Aitken *et al.* 1996), likely because bud set in saplings occurs in early summer, long before fall cold hardening. One might expect, however, that fall cold hardiness and bud set are more strongly associated in seedlings than in saplings, because bud set in seedlings occurs much later than in saplings, and closer to the initiation of fall hardening (Li and Adams 1993).

Therefore, the third objective of this chapter is to compare the relationships between cold hardiness and bud phenology in seedlings and saplings.

Materials and Methods

Materials

Materials for the sapling field trial and seedling nursery test were open-pollinated seeds (families) collected from forty parent trees in each of two low-elevation Douglas-fir breeding zones in western Oregon - a breeding zone west of Corvallis in the Coast Range, and a breeding zone northeast of Corvallis on the west slope of the Cascade Mountains. The 40 families within each zone were chosen from among the families represented in progeny field tests (20 families from each of two 30-family sets), based on stored seed availability for the nursery test. The experimental design, cold hardiness testing procedures, and shoot phenology assessments were described previously for the saplings (Aitken and Adams 1995a,b, 1996, 1997) and seedlings (see Chapter 2). Parent tree locations and environments are described in Balduman *et al.* 1999. Thus, only a summary of the methods will be provided in this chapter.

Sapling field tests

Progeny field tests were established in 1987 at seven sites in the Coast breeding zone and at six sites in the Cascade breeding zone using 1-0 seedlings. Families were sampled at one high and one low elevation test site, and in two years, in each zone in the sapling cold hardiness study in order to evaluate the consistency of family ranking for cold hardiness and phenology traits in different environments (Aitken and Adams 1996, 1997). The experimental design in the Coastal zone was a split plot, with 30-family sets as main plots, and families within sets represented by four-tree non-contiguous subplots. Four replications (blocks) of this design were found at the low elevation site and five at the high elevation site. In the Cascade zone, each 30-family set was planted as a separate randomized complete block design with five replications. Within each replication, families were represented by four-tree non-contiguous plots. By age seven (1992), when cold hardiness assessments were begun, survival at the four test sites averaged 91% (range 80 - 95%).

Seedling nursery test

Stratified seed from the 80 families (40 per breeding zone) were sown at 8x10 cm spacing into two raised nursery beds in Corvallis, Oregon, in April, 1995 (see Chapter 2). The experimental design was a split plot with four randomized complete blocks. The main plots were contrasting soil moisture treatments (well-watered *versus* moderate drought) applied during the second summer after sowing. Each main plot was subdivided into two replicate sub-blocks (sub-blocks A and B), each containing the 80 families, randomly allocated to 4-tree row-plots. Replicate sub-blocks were required in order to accommodate the large number of shoot samples needed for cold hardiness testing. In total, 4 seedlings/family-row subplot x 80 family-row subplots/sub-block x 2 sub-blocks/moisture regime x 2 moisture regimes/block x 4 blocks = 5120 test seedlings were established.

Seedling survival at the end of the experiment was 98%, however, symptoms of Lygus bug (*Lygus hesperidus* Hahn) attack (deformed and scarred apical shoots and buds) appeared on 4% of the seedlings at the end of the first summer (1995), and a natural frost in early November 1995 killed the apical bud of 28% of the seedlings (see Chapter 2). Artificial freeze tests were applied to non-damaged lateral shoots after the second growing season (i.e., 1996). The natural frost event in fall 1995 is not expected to have affected cold hardiness or bud phenology of non-damaged shoots the following year (pers. comm. Les Fuchigami, Oregon State University). Three observations support this opinion. First, all seedlings, including those which were heavily damaged, produced healthy foliage on non-damaged branches in the spring of 1996. Second, buds which developed on damaged seedlings in the fall of 1996 were large and healthy, and formed within the usual bud set period. Third, seedlings did not appear to sustain any natural frost injury during the winter of 1996-7.

Measurements

Methods of artificial freeze testing (AFT), and assessment of bud phenology were similar at the two ages. Cold hardiness testing in the field test was performed on all saplings after the sixth and seventh growing seasons: i.e., on five fall dates (September, October and November of 1992, and September and October of 1993), one winter date (January 1993), and three spring dates (March and April 1993, and April 1994). Cold hardiness testing in the nursery experiment was performed on all seedlings in one sub-block of each main plot (i.e., on 16 seedlings per

family in each moisture treatment), four times after the second growing season: i.e., on three fall dates (September, October and November of 1996), and one spring date (March of 1997).

Cold hardiness testing on each test date (month) began with cutting a 5 cm shoot tip from two lateral branches of each tree. Samples were individually labeled and transported on ice to the freeze-testing laboratory. The two shoot samples from each tree were frozen at different test temperatures in a programmable freezer. The AFT was initiated by subjecting the samples to -2 °C for 7-10 h, then lowering the temperature by 3-5 °C per hour until the test temperature was reached. After one hour at the test temperature, samples were placed in a cooler (2 °C) for at least 6 hours to thaw, and then placed on laboratory benches at room temperature for 6-8 days to allow symptoms of cold injury to develop.

Cold injury was assessed visually by recording for needles, stems and buds, the percentage of discolored tissue to the nearest 10%. The goal of AFT was to achieve intermediate levels of mean freeze injury to the shoot tissues (i.e., 30-70% tissue injury), so that family differences in cold injury would be maximized (Aitken and Adams 1996, and Chapter 2 of this thesis). To increase the likelihood of achieving this goal, two test temperatures were applied to all seedlings sampled on each test date (one temperature to each replicate shoot cutting). Test temperatures were selected on the basis of preliminary artificial freeze tests (AFTs) performed the week prior to the experimental AFTs. Temperatures selected for the experimental AFTs ranged from -8 and -12 °C in September in the seedling test to -35 and -40 °C in January in the sapling tests. Intermediate injury levels were typically achieved at one or both temperatures, and because family differences were greatest when injury scores at the two temperatures were averaged, mean scores were utilized in all analyses (Aitken and Adams 1996, 1997, and Chapter 2 of this thesis).

Sapling data from both the high and low elevation field sites were used because G x E interaction effects for cold injury were generally non-significant in saplings (Aitken and Adams 1996, 1997), and using data from both sites for both populations maximized the precision of family mean cold injury estimates. However, cold injury scores from only one fall and one spring test date at each age were considered, because of the need to simplify analyses, and because genetic correlations of cold injury scores between months in the same season were strong in both saplings (Aitken and Adams 1996, 1997) and seedlings (see Chapter 2). October AFT scores (i.e., October 1992 for saplings and October 1996 for seedlings) were used to assess

fall cold hardiness because estimates of individual heritabilities were highest at both ages in October. Sapling heritabilities for spring cold injury were greatest in April, so AFT scores for April 1993 were used for saplings. Spring cold hardiness in seedlings was represented by March 1997 AFT scores - the only spring month in which seedling cold hardiness was tested.

To further simplify analyses and to evaluate the feasibility of early testing for cold hardiness, data from seedlings only in the wet moisture regime were used. Genetic correlations for AFT traits between moisture regimes were strong ($\bar{r}_A$ = 0.89 - see Chapter 2), and using only the seedlings in the wet treatment provided a sample size (i.e., 16 seedlings/family) close to that commonly used in seedling genetic tests. Also, previous analyses have shown that 16 seedlings/family was clearly sufficient to reliably rank families for cold hardiness. Furthermore, a uniformly wet nursery environment is easier to maintain, and provides stronger heritabilities ($\bar{h}_i^2$ = 0.49) for AFT traits than a uniformly dry environment ($\bar{h}_i^2$ = 0.36) (see Chapter 2). As a final simplification, only damage to stems was considered, primarily because at both ages, heritabilities of AFT injury scores were stronger for stems than for needles or buds, and because genetic correlations between stems and the other two tissues were always positive, and in most cases were greater than 0.50 in fall tests, and greater than 0.80 in spring tests (Aitken and Adams 1996, 1997, and Chapter 2).

Bud phenology observations were made preceding each of the selected fall and spring cold hardiness periods indicated above (i.e., bud set in 1992 and bud burst in 1993 in the saplings; bud set 1996 and bud burst in 1997 in the seedlings). Bud set (BS) and bud burst (BB) were recorded bi-weekly on the terminal bud of a single marked secondary shoot of all trees in the sapling test, and on the apical bud of all seedlings in the nursery test. Buds were scored as either 'set' (smooth, well-developed, brown scales visible) or 'burst' (new needles visible) on each assessment, and the date of bud set and bud burst were estimated as the Julian dates on which these events were first noted. When lammas growth (second flushing) was observed (i.e., on less than 1% of the saplings, and on 14% and 12% of the Coast and Cascade seedlings, respectively), bud set was recorded as the date of the last bud set. Seedling bud set date was recorded on all seedlings in both replicate sub-blocks (i.e., on 32 seedlings per family), but bud burst was recorded on only one sub-block in each main plot (i.e., on 16 seedlings per family), because one sub-block in each main plot was harvested for a companion biomass study in the winter of 1996.

Statistical analysis

Data for each breeding zone and assessment age were analyzed separately. All analyses were conducted on single-tree observations. All variables were assumed to be random in both seedling and sapling analyses.

Statistical models and analysis of sapling traits and fall cold injury (FCI) and spring cold injury (SCI) in the seedling test were described in detail previously (Aitken and Adams 1996, 1997 and Chapter 2). The main difference between the experimental design of the Coast and Cascade breeding zone field sites was that family sets were nested within blocks at the Coast sites, while blocks were nested within sets at the Cascade sites. In the nursery experiment, families were not blocked by family sets, and preliminary analyses indicated that the 20-family sets did not differ statistically within each zone (see Chapter 2). Thus, family sets were ignored in the seedling analyses. FCI and SCI scores in saplings were subjected to arcsine square root transformation prior to analysis. Residual values were normally distributed in all other traits and, thus, were analyzed in the original scale.

All traits at the sapling stage utilized in this chapter were shown in earlier analyses to vary significantly ($p < 0.05$) among families (Aitken and Adams 1995a, 1996, 1997; Aitken and Adams, unpublished). Earlier analyses also provided restricted maximum likelihood (REML) estimates of variance components for sapling-age traits using the SAS VARCOMP procedure (SAS Institute Inc. 1996). Variance components of all seedling traits were obtained using the REML estimator in the SAS MIXED procedure, and family differences were tested using appropriate F-tests in the GLM procedure. Family differences were also significant for all seedling traits (Chapter 2).

The similarity of genetic control of cold hardiness and bud phenology traits at the two ages was evaluated by comparing estimates of individual heritabilities of the corresponding traits and genetic correlations between them (Objective 1 and 3). Likewise, genetic correlations between seedlings and saplings for each trait were estimated to evaluate genetic relationships between traits at the different ages (Objective 2). In all cases, the additive genetic variance (σ_A^2) was estimated as $3\,\sigma_F^2$ (where σ_F^2 is the family (seedling) or family within set (sapling) variance component), because open-pollinated Douglas-fir progeny are expected to be more closely related than half-sibs (Squillace 1974; Campbell 1979). Individual heritability estimates for FCI

and SCI in saplings were obtained from previous reports (Aitken and Adams 1996, 1997). Individual heritability estimates of BS and BB in saplings were estimated from variance components provided by Aitken and Adams (1997 and unpublished data). Individual heritabilities for sapling traits were estimated as:

$$[1] \qquad h_i^2 = \frac{\sigma_A^2}{\sigma_F^2 + \sigma_{FT}^2 + \sigma_e^2 + \sigma_w^2}$$

where

σ_{FT}^2 is the family-by-test site interaction,

σ_e^2 is the plot error (family-by-block interaction), and

σ_w^2 is the within family-plot error.

To estimate h_i^2 for seedling traits, equation [1] was modified by setting $\sigma_{FT}^2 = 0$. To be consistent (in terms of the number of observations per family) in the analysis of all seedling traits in this Chapter and all traits in Chapter 2, BS, which was recorded in both sub-blocks, was analyzed separately by sub-block, and the two resulting individual heritability estimates averaged. Standard errors of heritability estimates were calculated according to Dickerson (1969, pages 49-50), using the asymptotic variances of variance components derived in the REML procedure.

Genetic correlation estimates were obtained in two ways. When measurements of both traits were made on the same individuals, type A genetic correlations were calculated as:

$$[2] \qquad r_A = \frac{Cov_{F_{1,2}}}{\sqrt{\sigma_{F_1}^2 \times \sigma_{F_2}^2}}$$

where $\sigma_{F_1}^2$ and $\sigma_{F_2}^2$ are the estimated family (or family within set) variances of traits 1 and 2, and $Cov_{F_{1,2}}$ is the estimated family covariance between traits 1 and 2. In both seedlings and saplings, $Cov_{F_{1,2}}$ was solved from the equation:

[3] $$\sigma^2_{F_{1+2}} = \sigma^2_{F_1} + \sigma^2_{F_2} + 2(Cov_{F_{1,2}})$$

where $\sigma^2_{F_{1+2}}$ is the family variance of the sum of the values for traits 1 and 2.

Type B genetic correlations (r_B) were calculated to evaluate genetic relationships between traits measured on different individuals in the same families. This occurred when seedlings were measured for different traits in different replicate sub-blocks of the same main plot, or when measurements were made at different ages. To calculate r_B, $Cov_{F_{1,2}}$ in equation [2] was replaced by $Cov_{\bar{F}_{1,2}}$, the covariance of family means for traits 1 and 2.

Assessment of BS on seedlings in both sub-blocks of each main plot allowed both type A and B genetic correlations to be estimated between seedling BS and the other seedling and sapling traits. The two genetic correlations (r_A and r_B) were averaged to improve the precision of the correlation estimates.

The potential for early testing of cold hardiness, or *vice versa*, the potential response in seedling cold hardiness following selection for cold hardiness in saplings, and response in cold hardiness if bud phenology is selected as a surrogate for cold hardiness assessment, were evaluated by calculating expected correlated responses to selection (Falconer 1986, p. 286). It was assumed in the selection scenario for all traits that the 'best' 20% of parent trees in each breeding zone are selected on the basis of the performance of their open-pollinated progeny (e.g., those with the lowest mean cold injury, earliest BS or latest BB) at either the seedling or sapling stages. The selected parents are then placed in a clonal seed orchard to produce offspring by random mating. Expected correlated response (*CR*) in the seed orchard offspring was estimated according to Shellbourne (1969):

[4] $$CR_y = 2i(h^2_{f_x})^{1/2}(h^2_{f_y})^{1/2} r_{A_{x,y}} \sigma_{\bar{p}_y}$$

where

i = the selection intensity expressed in standard deviations = 1.40,

$h^2_{f_x}$ and $h^2_{f_y}$ are estimated family heritabilities for the selected (x) and response (y) traits,

$r_{A_{x,y}}$ is the estimated genetic correlation between the selected and response traits, and

$\sigma_{\bar{p}_y}$ is the estimated phenotypic standard deviation of family means for the response trait.

Family heritabilities for sapling traits (either x or y) were calculated as:

[5] $$h_f^2 = {}^{\frac{1}{4}}\sigma_A^2 \Big/ \sigma_{\bar{p}}^2$$

where

$$\sigma_{\bar{p}}^2 = \sigma_F^2 + {}^{\sigma_{FT}^2}\Big/_{t} + {}^{\sigma_e^2}\Big/_{tb} + {}^{\sigma_w^2}\Big/_{tbn}$$

and t is the number of sites (2); b is the number of blocks (4.5 in the coast zone (4 at the low elevation site and 5 at the high elevation site) and 5 in the Cascade zone); and n is the harmonic mean number of saplings per plot (estimated as 3.5 in the Coast and 3.2 in the Cascades). To estimate family heritabilities for seedling data, $\sigma_{FT}^2 = 0$, $t = 1$, $b = 4$, and $n = 3.9$. Family heritability for BS was analyzed separately by sub-block, and the two resulting individual heritability estimates averaged, as for individual heritability of BS (see above).

The expected response to direct selection (R_y) of the traits at each age was also calculated:

[6] $$R_y = 2i(h_{f_y}^2)\sigma_{\bar{p}_y},$$

and compared with the correlated response.

Direct and correlated responses for FCI and SCI at age 7 were back-transformed due to the use of arcsine square root transformed injury values in the estimation of $\sigma_{\bar{p}}$ for these two traits.

Results and Discussion

Genetic control of cold hardiness and implications for selection

Family ranges for cold hardiness traits were large for both seedlings and saplings, often exceeding 50% of the population mean (Table 3.1). Individual heritabilities were strong in both seedlings ($\overline{h_i}^2 = 0.60$) and saplings ($\overline{h_i}^2 = 0.49$), and similar trends in heritability estimates were observed for cold hardiness traits between seasons and breeding zones at both ages (i.e., at both ages heritability estimates were greater in spring ($\overline{h_i}^2 = 0.73$) than in fall ($\overline{h_i}^2 = 0.36$), and greater in the Coast breeding zone ($\overline{h_i}^2 = 0.69$) than the Cascade zone ($\overline{h_i}^2 = 0.40$)). Furthermore, strong genetic relationships were observed between seedling and sapling cold hardiness in both fall and spring (i.e., families that sustained little FCI (or SCI) at age 2 also sustained little FCI (or SCI) at age 7) (r_B was never < 0.78, Table 3.2), despite differences in the year of testing, growth environments and location, sampling design, and scoring personnel. These results attest not only to the similarity of the genetic regulation of cold hardiness at the different ages, but to the reliability of methods of artificial freeze testing and visual scoring to evaluate cold hardiness in both studies. The contention that seedling and sapling cold hardiness traits are controlled by similar sets of genes is also supported by the presence of similar correlations between spring and fall cold injury in seedlings and saplings (discussed below).

Direct selection for cold hardiness

Strong genetic control and significant family variation for SCI in both seedlings and saplings indicate that genetic improvement of spring cold hardiness would be highly effective at both ages, even under relatively weak selection intensity, as shown in expected response calculations (Table 3.3). For example, selecting 20% of the Coast parents with the most spring cold-hardy offspring (i.e., least cold injury scores) at age 2 is expected to reduce spring cold injury of 2-year-old seedlings in the next generation from 58.2 to 31.0% (i.e., a -27.2% response). Likewise, selection of Coast parents with the most fall cold-hardy offspring at age 2 is expected to reduce fall cold injury at age 2 by 34.2% in the next generation. Expected responses in fall and spring cold injury at age 2 were nearly as strong in the Cascade population (-26.9 and -21.1%, respectively). Expected responses in spring cold injury at age 7 from selection at the same age were of similar magnitude as responses of spring cold injury at age 2 (although somewhat weaker in the Cascade (-14.4%) population than in the Coast (-29.4%)). Expected responses to direct selection at age 7 for fall cold injury, however, were considerably smaller (-3.2% for Coast, and

Table 3.1. Estimated population means, range of family means, individual (h_i^2) and family (h_f^2) heritabilities, and family mean phenotypic variances (σ_P) for fall (FCI) and spring (SCI) cold injury after artificial freeze testing, and timing (Julian date) of bud set (BS) and bud burst (BB), at ages 2 and 7 in Coast and Cascade populations of coastal Douglas-fir.

Trait	Coast					Cascade				
	Pop. mean	Range of fam. means	h_i^2 [a]	h_f^2 [b]	σ_P	Pop. mean	Range of fam. means	h_i^2 [a]	h_f^2 [b]	σ_P
Age 2										
BS	245.3	225.0 - 260.4	0.30 [c]	0.47 [c]	80.4	240.1	226.2 - 256.3	0.32 [c]	0.51 [c]	71.9
FCI	69.4	33.7 - 97.2	0.66	0.56	265.7	50.5	23.3 - 84.0	0.35	0.46	204.5
BB	114.9	104.1 - 123.4	1.00 [d]	0.67	18.1	112.5	104.0 - 119.3	0.78	0.62	12.1
SCI	58.2	40.4 - 88.1	0.83	0.62	153.0	64.5	48.3 - 86.8	0.55	0.59	97.8
Age 7										
BS [e]	132.3	127.1 - 146.4	0.54	0.66	12.0	130.4	127.6 - 134.5	0.30	0.47	2.8
FCI [f]	24.6	12.8 - 39.8	0.26	0.56	0.007	23.9	7.6 - 58.6	0.16	0.38	0.006
BB [e]	134.8	121.6 - 145.8	1.00 [d]	0.70	16.1	133.5	126.2 - 137.0	0.70	0.65	6.2
SCI [f]	43.4	5.1 - 88.3	1.00 [d]	0.70	0.060	53.1	23.8 - 83.8	0.54	0.63	0.031

[a] Average standard error of h_i^2 = 0.16 (range 0.07 - 0.29).

[b] Average standard error of h_f^2 = 0.23 (range 0.23 - 0.25).

[c] Values were estimated separately by sub-block and then averaged.

[d] Estimate exceeded 1.00.

[e] BS values are for 1992; BB values are for 1993.

[f] Heritabilities and phenotypic variances based on arcsine transformed injury scores; means and family ranges based on original injury score.

Table 3.2. Estimated genetic correlations among stem cold hardiness (fall cold injury (FCI) and spring cold injury (SCI)), and bud phenology (Julian dates of bud set (BS) and bud burst (BB)) traits at age 2, age 7, and between ages 2 and 7, for two western Oregon breeding populations (Coast and Cascade) of Douglas-fir.

	Coast				Cascade			
age 2 \ age 2	**BS**	**FCI**	**BB**	**SCI**	**BS**	**FCI**	**BB**	**SCI**
BS		0.96	0.60	-0.52		0.65	0.19	0.03
FCI			0.75	-0.54			0.11	0.08
BB				-0.90				-0.82
SCI								
age 7 \ age 7	**BS**	**FCI**	**BB**	**SCI**	**BS**	**FCI**	**BB**	**SCI**
BS		0.28	0.88	-0.85		0.38	0.90	-0.96
FCI			0.46	-0.36			0.48	-0.14
BB				-0.94				-0.90
SCI								
age 7 \ age 2	**BS**	**FCI**	**BB**	**SCI**	**BS**	**FCI**	**BB**	**SCI**
BS	0.76	0.70	0.79	-0.49	0.11	0.29	1.00[a]	-0.70
FCI	0.47	0.80	0.64	-0.38	0.56	1.00[a]	0.42	-0.17
BB	0.81	0.87	0.93	-0.73	0.11	0.26	0.91	-0.67
SCI	-0.71	-0.71	-0.88	0.87	-0.09	-0.08	-0.76	0.78

[a] Genetic correlation exceeded 1.00.

Table 3.3. Direct (bold) and correlated responses expected in fall (FCI) and spring (SCI) cold injury scores at ages 2 and 7 when 20% of parent trees are selected on the basis of the performance (i.e., low FCI and SCI, early BS (bud set) and late BB (bud burst)) of their open-pollinated offspring.

Selection			Response [a]			
			Coast		Cascade	
Trait	Age	Age	FCI	SCI	FCI	SCI
BS	**2**	**2**	-27.9	11.5	-17.1	-0.5
FCI	**2**	**2**	**-34.2**	14.0	**-26.9**	-1.5
BB	**2**	**2**	28.1	-25.6	3.5	-17.8
SCI	**2**	**2**	19.3	**-27.2**	-2.4	**-21.1**
BS	**7**	**2**	-26.0	13.8	-7.9	13.2
FCI	**7**	**2**	-27.3	9.8	-24.8	3.0
BB	**7**	**2**	33.1	-21.1	8.4	-14.9
SCI	**7**	**2**	27.1	-25.3	2.7	-17.1
BS	**7**	**7**	-0.3	20.6	-0.3	10.2
FCI	**7**	**7**	**-3.2**	3.4	**-1.8**	0.2
BB	**7**	**7**	0.9	-26.2	0.7	-12.4
SCI	**7**	**7**	-0.5	**-29.4**	0.1	**-14.4**
BS	**2**	**7**	-1.2	4.5	-0.2	5.0
FCI	**2**	**7**	-2.0	3.7	-2.1	0.3
BB	**2**	**7**	2.9	-15.7	0.2	-6.5
SCI	**2**	**7**	1.8	-20.3	0.0	-8.3

[a] Negative response means reduced cold injury.

-1.8% for Cascade population), because family heritabilities were weak for this trait (Table 3.1). Low mean FCI scores in saplings may have contributed to the weak FCI heritability and limited the expression of family variation, emphasizing the importance of choosing test temperatures that inflict intermediate levels of mean cold injury.

Correlated responses in cold injury of saplings to selection at the seedling stage and vice versa

Regulation of cold hardiness traits by similar sets of genes in seedlings and saplings implies that selection for alleles which control hardiness at one age will also select alleles which control hardiness at the other age. For example, the expected response in spring cold injury of saplings in the Coast population is -29.4%, if selection is based on AFT at the same age, and -20.3% if selections are made at age 2 (Table 3.3). Expected direct and correlated responses in spring cold injury of saplings are about half the magnitude in the Cascade population (-14.4% and -8.3%, respectively). Expected responses of fall cold injury of saplings are poor, regardless of whether selections are made at age 2 or age 7. Expected responses in fall or spring cold injury of seedlings, however, are strong, and of similar magnitude, regardless of whether selections are made at the seedling or sapling stage.

Early selection can therefore be used to quickly and cheaply screen large numbers of individuals grown in common gardens or nurseries for cold hardiness at the seedling stage when trees are most frost susceptible, while assuring gains in hardiness at the sapling stage approach those that would be obtainable had selection been delayed to the later age. Alternatively, selection for cold hardiness in Douglas-fir progeny tests already established for tree improvement programs in the Pacific Northwest and elsewhere (e.g., Canada, France, Germany and New Zealand), would improve cold hardiness not only in older trees, but also at the seedling stage.

Indirect responses of cold hardiness in one season to selection for cold hardiness in the other season

The effect of selection for cold hardiness in fall or spring on cold hardiness in the other of these seasons was addressed for seedlings in Chapter 2. In this chapter, the impacts of indirect selection are explored more broadly, in particular, to include indirect responses at both ages, and indirect responses at one age when selection is applied at the other age.

In both seedlings and saplings, genetic correlations between fall and spring cold injury were negative and moderate (r_A = -0.54 and -0.36, ages 2 and 7, respectively) in the Coast population, but negligible (r_A = 0.08 and -0.14, ages 2 and 7, respectively) in the Cascade population (Table 3.2, and see Figure 3.1 - a scatterplot of fall *versus* spring family mean cold injury scores of the two populations). Consequently, in the Cascade breeding zone, fall and spring cold hardiness appear to be genetically independent of each other; if improvement is desired for both traits, both traits must be selected. However, selection for cold hardiness in one season will have no impact on cold hardiness in the other season.

In the Coast breeding zone, fall and spring cold hardiness are not independent: selection for one trait will have a detrimental impact on the other unless both traits are selected. This is illustrated most strongly at age 2, when direct selection for spring cold hardiness is expected to reduce spring cold injury by 27.2%, but will increase fall cold injury by 19.3% (Table 3.3). The detrimental response in FCI to selection for SCI is not as great at age 7 as it is at age 2 because the heritability for FCI and the FCI-SCI genetic correlation are weaker at age 7 than at age 2. Greater cold injury risk in spring than in fall in the Pacific Northwest (Timmis *et al.* 1994) helps to alleviate the potential negative impact of reduced fall cold hardiness in response to selection for spring cold hardiness. Independent culling (Zobel and Talbert 1984) or use of a selection index to select families will also help mitigate the antagonism caused by negative FCI-SCI genetic correlations. Despite the negative correlation between fall and spring cold hardiness in the coast population, there is considerable family variation for spring cold hardiness among the most fall cold-hardy families. Consequently, families which are cold hardy in spring and fall should not be difficult to identify.

It was expected that the more rapid change in minimum daily temperature in both fall and spring in the Cascade Mountains than in the Coast Range would result in smaller ranges in family means for BB and BS date, and consequently, in weaker family variation for FCI and SCI in the Cascade population, which in turn, would result in weaker FCI-SCI genetic correlations in the Cascade population. Family variation for FCI was nearly equivalent in both populations for seedlings (CV_F% = 20.3 - Coast, and 22.0 - Cascade) and saplings (CV_F% = 14.3 - Coast, and 12.2 - Cascade) (seedling values for CV_F% for October stem injury from Table 2.1; sapling values calculated from Aitken and Adams 1996 and 1997). However, family variation for SCI

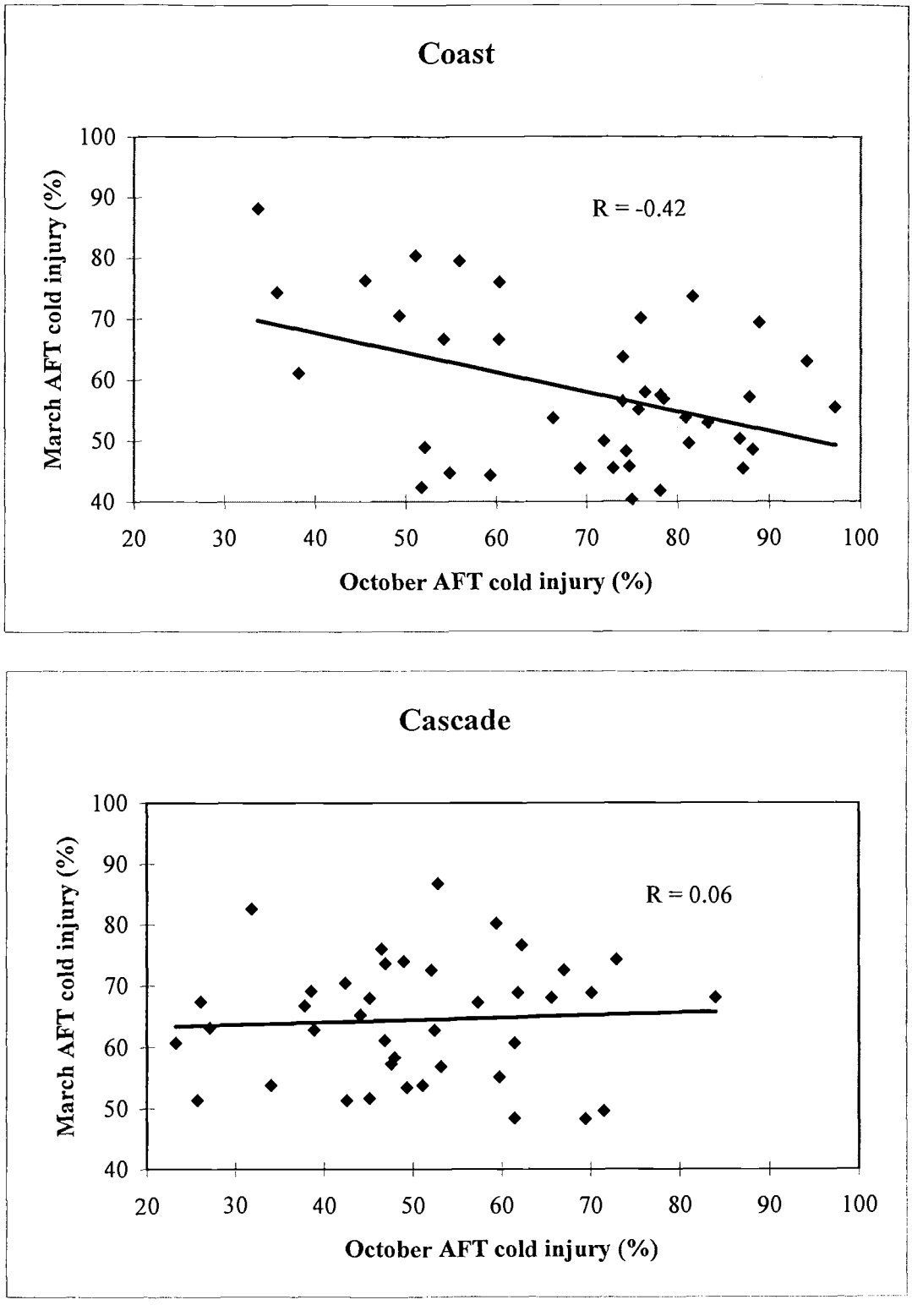

Figure 3.1. Scatterplot of fall (October) *versus* spring (March) family mean cold injury scores from artificial freeze tests of stem tissue of Coast and Cascade populations of Douglas-fir seedlings grown in the wet moisture regime.

was greater, as anticipated, in the Coast than the Cascade population, for seedlings ($CV_F\%$ = 19.3 - Coast, and 13.5 - Cascade) and saplings ($CV_F\%$ = 35.1 - Coast, and 21.7 - Cascade).

Expected correlated responses may also be used to examine indirect cold hardiness response in one season and age, to cold hardiness selection applied in the other season and age. Because of lack of correlation between FCI and SCI in the Cascade population, little response in one trait is expected if the other trait is selected, regardless of which age selections are made (Table 3.3). In the Coast population, however, detrimental responses (i.e., indirect impact on cold hardiness in the non-selected season) are nearly as great whether selections are made at age 2 or age 7.

Bud phenology as a surrogate for cold hardiness selection

Strong negative genetic correlations between BB and SCI were estimated for both seedlings (r_A = -0.90 for Coast and -0.82 for Cascade population) and saplings (r_A = -0.94 for Coast and -0.90 for Cascade population) (Table 3.2), indicating that BB timing can be an effective surrogate for spring AFTs at both ages. For example, selecting for delayed BB at age 2 (age 7) in the Coast population is expected to result in 25.6% (26.2%) less SCI in the next generation at age 2 (age 7). Direct selection for spring cold hardiness based on AFT is expected to be only marginally better at reducing SCI (i.e., 27.2% and 29.4% less SCI at age 2 and 7, respectively). Bud phenology also appears to be an effective predictor of fall cold hardiness in seedlings (r_A - BS-FCI = 0.96 for Coast and 0.65 for Cascade population), but not in saplings (r_A - BS-FCI = 0.28 for the Coast and 0.38 for the Cascade population). While selection for earlier BS in the Coast population at age 2 is expected to reduce FCI in the next generation by 27.9% (nearly as great as the -34.2% response from direct selection of FCI in AFTs), selection for earlier BS at age 7 in this population is expected to reduce FCI by only 0.3% (i.e., by only 10% of the 3.2% reduction in FCI expected from direct selection of FCI in AFTs). Expected results for indirect selection of spring and fall cold hardiness at both ages in Cached populations based on bud phenology are similar to those expected for the Coast population (Table 3.3).

An explanation of the difference between seedlings and saplings in BS-FCI genetic correlations likely resides in the striking difference in timing of bud set at the two ages: bud set in saplings occurred on May 12 and 10, 1992 (Coast and Cascade, respectively), far in advance of stem hardening in late summer or early fall. In contrast, bud set in seedlings occurred on September 2 and August 28, 1996 (Coast and Cascade respectively), closer to the time of stem

hardening. Considerably earlier bud set in 15-year-old saplings (June 3) than in two-year-old seedlings (September 6) was also documented in coastal Douglas-fir from another breeding zone in coastal Oregon (Li and Adams 1993). Failure of BS to predict fall cold hardiness in saplings may also be related to weak family variation for FCI, which may be an artifact of the use of sub-optimal test temperatures in the sapling fall cold hardiness test, as discussed above.

Drought not only narrowed the range among families in BS date (see above), it also hastened its onset. Average date of bud set occurred 9.1 (Coast) and 8.2 (Cascade) days earlier in the dry than the wet regime. Both of these factors likely contributed to the weaker genetic correlations between BS and FCI in the dry (r_A = 0.84 - Coast and 0.44 - Cascade) than in the wet (r_A = 0.96 - Coast and 0.65 - Cascade) regime. Consequently, if BS is to be used as a surrogate for fall AFT, it may be advantageous to employ a well-watered summer moisture regime in order to ensure that the range among families in seedling BS date is maximum, and that BS date approaches the date on which fall hardening begins.

It thus appears that bud phenology assessment provides a suitable surrogate for AFT cold hardiness evaluation for seedlings in fall and spring, and for saplings in the spring. AFTs require availability of a freezer with a precise, programmable temperature controller, but sampling can be done on a single day. Assessing bud phenology requires no specialized equipment, but repeated visits to nursery or field sites are necessary. Therefore, the choice of AFTs *versus* bud phenology assessment will likely depend on the availability of a freezer, and accessibility of nursery or field sites (Aitken and Adams 1997). The proximity of nursery test sites to research stations in many situations obviates significant travel considerations, and may make bud phenology the preferred technique for early testing of cold hardiness.

Chapter 4

Preliminary Results and Recommendations for the Assessment of Genetic Variation in Cold Hardiness Following Mid-winter Warming in Douglas-fir

Abstract

Global warming is expected to increase the incidence of brief periods of warm weather in mid-winter. Partial dehardening due to mid-winter warming, followed by a return to freezing conditions, could result in substantial cold injury to perennial plants. The objectives of this study were to examine the impact of winter-warming on cold hardiness in Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco), to evaluate the extent of genetic variation and control of cold hardiness after mid-winter warming, and to assess the degree to which cold hardiness after mid-winter warming is associated genetically with cold hardiness in other seasons.

Cold injury was scored visually after artificial freeze testing detached shoots of 40 open-pollinated families from each of two populations (Coast and Cascade) of coastal Douglas-fir in western Oregon in fall, winter, in winter after a brief period of artificial warming (11 days at 16-20 °C), and in spring. Stems dehardened to roughly the same level in the winter-warmed and spring assessments, although needles and buds retained more hardiness in the winter-warmed assessment. Nonetheless, genetic variation ($CV_F\%$) and individual tree heritabilities (h_i^2) for cold injury were weak in the winter (mean $CV_F\% = 6.8$, $\bar{h}_i^2 = 0.22$) and winter-warmed assessments (4.2 and 0.21), relative to the spring assessment (16.2 and 0.68). Direct response to screening or breeding for winter cold hardiness following warming is therefore expected to be weak. Genetic correlations of stem cold hardiness in the winter-warmed assessments with hardiness in the winter and spring assessments were moderate in the Coast population ($r_A = 0.68$ and 0.75, respectively) and weak in Cascade population ($r_A = -0.12$ and $= 0.15$, respectively), indicating that indirect responses in cold hardiness after winter warming would be small-to-moderate in the Coast population, but negligible in the Cascade population, following selection for increased winter or spring cold hardiness. Results in this study may have been affected by the choice of dehardening treatment and small samples sizes. These results also point to the need

to 1) use a range of dehardening temperatures and durations in dehardening treatments, 2) assess the impact of using detached shoots in dehardening experiments, and 3) examine the role of re-hardening in spring phenology.

Introduction

The threat of global warming is considered more serious for slowly colonizing, long-lived trees, than for rapidly colonizing, annual plants (Bolin *et al.* 1986). While substantial regional anomalies in all climatic components are anticipated with global warming (Schneider and Rosenburg 1988), one of the greatest potential threats for forest trees is premature dehardening (i.e., deacclimation) due to brief periods of unseasonably warm winter weather, which may leave trees susceptible to injury from subsequent winter or spring frosts (Cannell 1989a; Cannell and Smith 1986; Hänninen 1991, 1996; Guak *et al.* 1998).

A substantial loss of cold hardiness (critical temperatures rose from -75 °C to -35 °C) of 18 *Pinus sylvestris* (L.) clones in Sweden was noted in assessments made on 23 March, 1992, following unusually mild temperatures during early and mid-March of that year (Nilsson and Wilfridsson 1995). In British Columbia in 1989, January minimum daily temperatures 4.9 °C above normal were followed by a sudden severe frost (-30 °C), resulting in widespread frost injury to several tree species in (van der Kamp and Worrall 1990). Dieback of several deciduous species in Canada and the United States this century has been related to frost injury sustained following sudden and prolonged mid-winter thaws (Auclair *et al.* 1992). Mid-winter thaws of unprecedented magnitude have been documented in the northeastern United States and are the likely cause of dehardening and winter frost injury in *Picea rubens* Sarg. (Strimbeck *et al.* 1995). Reports such as these suggest that premature dehardening may be a subject of significant concern in forestry.

Timing of bud burst date is closely related to spring cold hardiness (later bud burst is associated with greater spring cold hardiness), and its ease of measurement has made it a putative surrogate for estimating relative spring cold hardiness ranking (Dormling 1982; Hannerz 1994a; Aitken and Adams 1997; and see Chapter 2). Once chilling requirements (approximately 80-90 days for coastal Douglas-fir - *Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco) have been met (which usually occurs by mid-January in western Oregon - Lavender and Stafford 1984), bud burst date is primarily heat sum dependent (Wareing 1956; Thomson and Moncrieff 1982; Hannerz 1999). That is, bud burst occurs when a genotype-specific heat sum requirement (HS_{req}) is met. (Failure to satisfy chilling requirements may result in higher HS_{req}, delaying the date of bud burst, and presumably the initiation of dehardening as well - Worrall and Mergen 1967;

Nelson and Lavender 1979; Campbell and Sugano 1979; Guak *et al.* 1998.) As a result, heat sums have been used extensively to model bud burst date in research aimed to understand or minimize cold injury in forestry (Campbell 1974; Campbell and Sugano 1975, 1979; Cannell 1983, 1989a,b; Hannerz 1994b, 1999; Kramer 1995; von Wuehlisch 1995). The heat sum on day n (HS_n) is the sum of the mean daily temperatures (T_m) above a threshold temperature (T_{th}, usually 2-6 ^{0}C) after a given start day in winter (Hannerz 1999; Worrall 1983):

$$[1] \quad HS_n = \sum_{1}^{n} (T_m - T_{th}) \cdot$$

Genetic differences in bud burst timing and spring cold hardiness (Campbell and Sugano 1975, 1979; White *et al.* 1979; Rehfeldt 1983a; Adams and Bastien 1994; Aitken and Adams 1997) are usually attributed to genetic differences in HS_{req} (Campbell and Sugano 1975, 1979; Skuterud and Dietrichson 1994; von Wuehlisch *et al.* 1995), although genetic variation in threshold temperature may also influence bud burst timing and dehardening by affecting the rate of heat sum accrual (heat sums accrue faster in individuals with lower threshold temperatures) (Campbell and Sugano 1975; Worrall 1975, 1983; Kramer 1995; Hannerz 1999). Dehardening was also found to occur faster at higher temperatures and at later stages of quiescence in *Cornus sericea* (Kobayashi *et al.* 1983), although the mechanism - lower threshold temperatures or lower heat sum requirement for dehardening - was not determined. And finally, decreasing (colder) T_{th} and HS_{req} with increasing elevation and continentality (Campbell and Sugano 1979; Worrall 1983, 1993) have been ascribed to adaptations to shorter growing season (Worrall 1983), and suggest that high elevation, continental provenances may be most susceptible to cold injury following a brief period of winter warming.

Given that periods of mid-winter warming may become more prevalent with global warming, it is important to determine whether there is genetic variation for cold hardiness under these conditions. Thus, the main objective of the study described in this chapter was to evaluate the extent of genetic variation and control of cold hardiness after mid-winter warming in coastal Douglas-fir. Temperate perennial plants are most susceptible to frost damage in early spring, just prior to, and after, bud burst, and in early fall. Thus, genetic (family) variation in fall and spring cold hardiness of Douglas-fir seedlings, using artificial freeze testing (AFT) of detached shoots was the primary focus of the research reported in this thesis (Chapters 2 and 3). Family variation

in cold hardiness after mid-winter warming was investigated with the same materials employed in Chapters 2 and 3, by harvesting terminal shoots of seedlings in February and then subjecting them to AFT after a brief period of warming.

A second objective of this study was to evaluate the degree to which genetic control of cold hardiness after mid-winter warming is associated with cold hardiness in fall, mid-winter (prior to warming) and spring. In particular, it was of interest to evaluate the implications of selection for cold hardiness in fall, winter and spring on cold hardiness in winter after warming.

Materials and Methods

Test materials and design

Details concerning the populations, planting design, seedling culture, moisture regimes, artificial freeze testing and visual cold injury scoring procedures are provided in Chapter 2. Forty open-pollinated families of Douglas-fir from each of two western Oregon breeding populations (one from a breeding zone in the Coast mountains and one from a breeding zone on the west slope of Cascade mountains) were grown for two years in raised nursery beds in Corvallis, Oregon. Seed were sown in April 1995 in a split-plot design containing four randomized complete blocks. Main plots were two moisture regimes, which were applied in the second growing season (1996): well-watered (wet) and moderate drought stress (dry). Within each main plot, all 80 families were randomized within each of two replicate sub-blocks (A and B). Each family was represented by a four-tree row sub-plot in each sub-block. Seedling survival at the end of the experiment was 98%.

Cold hardiness was assessed in October 1996 ('fall'), early February 1997 ('winter'), and in late March 1997 ('spring'). Cold hardiness was also assessed in mid-February 1997 ('winter-warmed') after a period of warming. To ensure that family differences in dehardening in the winter and winter-warmed assessments were not confounded with insufficient chilling, these assessments were performed in February, well after chilling requirements for bud burst had been attained by all families.

Artificial freeze testing of detached shoots is a well established means of assessing cold hardiness in forest trees (Timmis and Tanaka 1976; Sakai 1982; Burr *et al.* 1990; Raymond *et al.* 1992; Aitken and Adams 1995a; Johnson and Hirsh 1995), and has also been used to assess the impacts of mid-winter warming on stem dehardening (Dietrichson 1993; Skuterud and Dietrichson 1994). Two lateral shoots (branches) from each seedling in sub-block A were used for fall cold hardiness assessments, and from sub-block B for spring cold hardiness (Chapter 2). Seedlings in sub-block A were also used for the winter and winter-warmed assessments, but because there were insufficient lateral shoots available after the September and October artificial freeze tests (AFTs) (see Chapter 2), the apical portion (8-12 cm) of the primary shoots (i.e., the 1996 leader) were assessed for cold hardiness in the winter and winter-warmed assessments. (See Table 4.1 for a summary of the sampling and AFT procedures for the four assessments.) Shoots for the winter and winter-warmed assessments were sampled at the same time, with leader shoots of two trees in each family sub-plot destined for each assessment. Thus, each family was represented by 2 cuttings from each of 8 sub-plots (4 sub-blocks in each of the wet and dry moisture treatments).

To accommodate freezer space limitations, shoot harvesting for the winter and winter-warmed assessments was performed in two lots, each consisting of two of the experimental blocks. Harvesting and cold hardiness processing for the winter assessment required 3 days for each lot, the first lot beginning February 1 and the second lot a week later. The ends of all cut shoots were immediately immersed in water and maintained at 2 °C until harvesting of each lot was completed. Upon completion of harvesting, two randomly selected shoots from each 4-tree family sub-plot were subjected to AFT (winter assessment). The two remaining shoots, retained for the winter-warmed assessment, were placed in 500 mL plastic buckets containing 250 mL of deionized water (20 shoots per bucket), with the cut surface immersed and the apical bud exposed to natural lighting, and kept in a warm greenhouse (20/16 °C day/night; 50% relative humidity).

Detached shoots in the winter-warmed assessment received a heat sum of 143 degree-days during the 11 day warming treatment in the greenhouse (calculated using T_{th} = 5 °C), when their cold hardiness was also assessed by AFT. The heat sum employed in the winter-warmed assessment was chosen on the basis of heat sums (calculated from weather records - Oregon

Table 4.1. Summary of the sampling and artificial freeze test procedures for fall, winter, winter-warmed and spring cold hardiness assessments.

Assessment	Sampling date	shoot type tested	Cuttings per seedling	Seedlings sampled in each family sub-plot	Obs. per fam. sub-plot	Sub-block sampled	Moisture treatment sampled	Obs. per assessment[a]	Test temp. (°C)		Data selected[b]					
											Coast			Cascade		
									T1	T2	Needles	Stem	Bud	Needles	Stem	Bud
Fall	10/20/96	lateral	2	4	4	A	wet	16	-12.5	-15.5	T12	T12	T12	T12	T12	T12
Winter	2/1/97	apical	1	2	1	A	dry, wet	8	-33.0	-39.0	T1	T2	---[c]	T1	T1	T12
W-W[d]	2/1/1997[e]	apical	1	2	1	A	dry, wet	8	-16.0	-20.0	---	T12	---	T12	T2	---
Spring	03/24/97	lateral	2	4	4	B	wet	16	-15.0	-19.0	T12	T12	T12	T12	T12	T12

[a] Number of observations per assessment = number of observations per family sub-plot x 4 blocks x number of moisture regimes sampled.

[b] i.e., Data from this test temperature was used in the analysis; T12 = average injury score from test temperatures 1 and 2.

[c] family variation non-significant at T1, T2 and T12.

[d] W-W = winter-warmed.

[e] Artificial freeze testing took place 11 days later, after the warming treatment (temperature 16/20 °C day/night x 11 days).

Climate Service, Oregon State University) used in previous studies in which an intermediate level of dehardening was achieved (Schuch *et al.* 1989b; Aitken and Adams 1997).

Artificial freeze tests

Shoots were wrapped first with cheesecloth moistened with tap water, and then with aluminum foil to keep the shoots moist during freeze testing and subsequent injury symptom development. The packets were placed in a Forma Scientific freezer, model 8270/859M, with a West M3750 temperature controller, and temperatures lowered to the test temperature at a rate of 3-5 °C/h, and maintained at the test temperature for one hour, whereupon the packets were removed and placed in a refrigerator (2 °C) for at least 7-10 hours to thaw. Once thawed, the packets were transferred to laboratory benches (16-18 °C) where they were held for 6-8 days in order for signs of cold injury to develop. Cold injury to needles, stem (phloem) and buds (meristem needle primordia) was visually evaluated through a magnifying lens. Injury to each tissue was recorded to the nearest 10% as the percentage of tissue that was damaged.

Family variance for cold injury varies with test temperature, there being a single test temperature at which family variance is maximum (usually occurring at test temperatures that cause mean intermediate levels of injury - i.e., 30-70% tissue damage). To better ensure that intermediate levels of damage were achieved, two freeze test temperatures were applied on each test date (assessment). The two test temperatures for each AFT (i.e., each assessment) were selected by interpolating from preliminary AFTs the test temperatures that would result in 40% and 60% tissue injury, respectively. The following test temperatures were utilized: -12.5 and -15.5 °C (fall), -33 and -39 °C (winter), -16 and -20 °C (winter-warmed), and -15 and -19 °C (spring). In the fall and spring assessments, one shoot from each seedling was subjected to each test temperature. In the winter and winter-warmed assessments, one leader from each family sub-plot was subjected to each test temperature (see Table 4.1).

Statistical analysis

All analyses were performed separately by breeding zone. To determine which data (i.e., scores from which test temperatures) provided the greatest ability to discriminate among families, preliminary analyses examined family variation in cold injury at the two test temperatures (T1 and T2) separately, and the mean injury scores (T12) averaged across both test temperatures (T1 and T2) (see Chapter 2). For each test date x tissue combination, the data

resulting in the largest family variation was chosen for subsequent analyses (see Table 4.1). Residual values of all cold hardiness traits were normally distributed and, thus, were analyzed in the original scale.

For analysis of cold hardiness traits in fall and spring, only data from the wet moisture regime was utilized. This gave 16 seedlings/family, which is consistent with sample sizes normally used in seedling studies (see Chapter 3 and Table 4.1). However, in order to have large enough samples of cuttings per family in the winter and winter-warmed assessments, trees from both moisture regimes (treatments) were required. In previous analyses of spring and fall cold injury traits (Chapter 2), family x moisture interactions were weak or non-significant ($p > 0.05$) and genetic correlations between cold injury in wet and dry regimes were strong ($\bar{r}_B > 0.86$), with few exceptions. In addition, family x treatment interaction was nearly always non-significant in preliminary analyses of cold injury scores in the winter and winter-warmed assessments. Moisture regimes were therefore ignored in analyses of cold injury during these two assessment periods, and the 4 blocks in each moisture regime were pooled into 8 total blocks. Thus, in the winter and winter-warmed assessments, each family was represented by 8 observations (one observation per family sub-plot x 8 blocks).

To test the significance of family differences in cold injury scores in the winter and the winter-warmed assessments, ANOVA was performed in the SAS GLM procedure (SAS Institute 1996) using type III sums of squares. The following linear model was used to represent individual seedling values for each assessment:

[2] $$Y_{ij} = \mu + B_i + F_j + e_{ij}$$

where Y_{ij} is the cold injury score for the jth family in the ith block,

μ is the overall mean,

B_i is the random effect of the ith block, with $E(B_i) = 0$, and $var(B_i) = \sigma_B^2$,

F_j is the random effect of the jth family, with $E(F_j) = 0$, and $var(F_j) = \sigma_F^2$,

e_{ij} is the experimental error, the random interaction of blocks and families, with $E(e_{ij}) = 0$, and $var(e_{ij}) = \sigma_e^2$.

An additional term, random within-plot error, e_{ijk}, was added to the model in the evaluation of fall and spring cold hardiness to account for the multiple observations within a family sub-plot (Chapter 2). Variance components were estimated with the same models, using the restricted maximum likelihood (REML) estimator in SAS MIXED procedure (SAS Institute Inc. 1996).

The strength of genetic control of each of the traits was estimated with individual tree heritabilities (h_i^2) to assess the potential for each trait (in particular, winter cold hardiness following warming) to respond directly to artificial selection, or indirectly through selection for a correlated trait. Individual heritabilities for cold injury in the winter and winter-warmed assessments were estimated as:

$$[3] \qquad h_i^2 = \frac{3\sigma_F^2}{\sigma_F^2 + \sigma_e^2}.$$

The variance component for within plot error, e_{ijk}, was added to the denominator of equation [3] in the estimation of individual heritability of fall and spring cold hardiness. The additive genetic variance (numerator of the h_i^2 equation) was estimated as three (rather than four) times the family variance, as open-pollinated progeny are more closely related than half-sibs (Squillace 1974; Campbell 1979). Standard errors of heritabilities were calculated according to Dickerson (1969, pages 49-50) using the asymptotic variances of variance components derived in the SAS VARCOMP procedure.

To evaluate the degree of genetic inter-relationships between cold hardiness traits and the influence that selection for fall, winter, or spring cold hardiness might have on mid-winter hardiness after warming, genetic correlations were estimated. When both traits were measured on the same individuals (e.g., fall and winter cold injury), or between different individuals in the same family sub-plot (winter and winter-warmed cold injury), type A genetic correlations (r_A) were estimated as:

$$[4] \qquad r_A = \frac{Cov_{F_{x,y}}}{\sqrt{\sigma_{F_x}^2 \times \sigma_{F_y}^2}}$$

where $\sigma^2_{F_x}$ and $\sigma^2_{F_y}$ are the estimated family variances of traits x and y, respectively. $Cov_{F_{xy}}$, the estimated family covariance between traits x and y, was calculated from:

$$[5] \qquad Cov_{F_{xy}} = (\sigma^2_{F_{x+y}} - \sigma^2_{F_x} - \sigma^2_{F_y})/2$$

where $\sigma^2_{F_{x+y}}$ is the estimated family variance of the sum of the values for traits x and y for each seedling.

When both traits were measured on individuals from the same family in different sub-blocks (e.g., fall and spring cold hardiness), type B genetic correlations (r_B, Burdon 1977) were estimated:

$$[6] \qquad r_B = \frac{Cov_{\bar{F}_{x,y}}}{\sqrt{\sigma^2_{F_x} \times \sigma^2_{F_y}}}$$

where $Cov_{\bar{F}_{xy}}$ is the covariance of family mean scores for traits x and y, and

$\sigma^2_{F_x}$ and $\sigma^2_{F_y}$ are the family variances of traits x and y, respectively.

(Note: type B genetic correlations are generally reserved for those situations in which different traits are measured on different individuals within a family, as in the case of the correlation between the winter and winter-warmed assessments. However, in the correlation of these two traits, the type B estimate may be upwardly biased because of environmental covariance (Falconer 1986, pages 282-283) between observations of the two traits within each block. In this situation, type A correlations may be calculated because these traits were measured on different trees within the same family sub-plot. That is, family covariances can be estimated from analyses of covariance based on sub-plot means. Genetic correlation estimates between winter and winter-warmed assessments were similar using type A and B methods, indicating that environmental correlation was weak.)

Results and Discussion

The availability of a limited number of seedlings per family for this experiment imposed significant design constraints, which precluded making strong conclusions. Nonetheless, several valuable observations regarding mid-winter dehardening can be wrought from the results, and insights obtained for future investigations.

Tissue hardiness

Apical shoots were extremely cold hardy in early February, as evidenced by the very low mean test temperature (-36 °C) required to elicit intermediate levels of injury in the winter assessment (Fig. 4.1). Seven weeks later, at the end of March, seedlings had dehardened to the point that the mean test temperature necessary to cause intermediate levels of injury had risen to -17 °C, about mid-way to the -6 °C required to attain intermediate levels of injury in fully dehardened shoots (Timmis *et al.* 1994). Levels of stem cold hardiness in the winter-warmed and spring assessments were similar (Fig. 4.1), as may be expected from the fact that seedlings in the two assessments had received nearly identical amounts of warming (191 and 188 degree-days, respectively) (temperatures provided by the Oregon Climate Service, Oregon State University) at the time of artificial freeze testing. At the initiation of the warming treatment on February 1, seedlings in both assessments had received 48 degree-days (calculated beginning January 1, with T_{th} = 5 °C). Seedlings in the winter-warmed assessment accumulated the remaining degree-days in 11 days, while those in the spring assessment required 52 days. Similar levels of dehardening resulting from similar heat sums accumulated at different rates in this study, suggest that for stem tissue, heat sums accumulate as a linear response to temperature, at least during the early stages of dehardening, although faster rates of dehardening may be observed at high temperatures in the later stages of dehardening (Kobayashi *et al.* 1983). For future investigations, examination of cold hardiness following the application of a range of heat sum levels, each applied at a range of temperatures, would allow the effect of developmental stage and dehardening temperature on dehardening rate to be evaluated.

The other striking feature regarding cold hardiness in the winter-warmed assessment is that needles and buds were substantially hardier than stems in both populations (Fig. 4.1). This may be a reflection of faster dehardening rates in stems, or it may be an artifact of the use of detached leaders (e.g., stem tissue may re-hydrate faster than needles or buds when stems are detached).

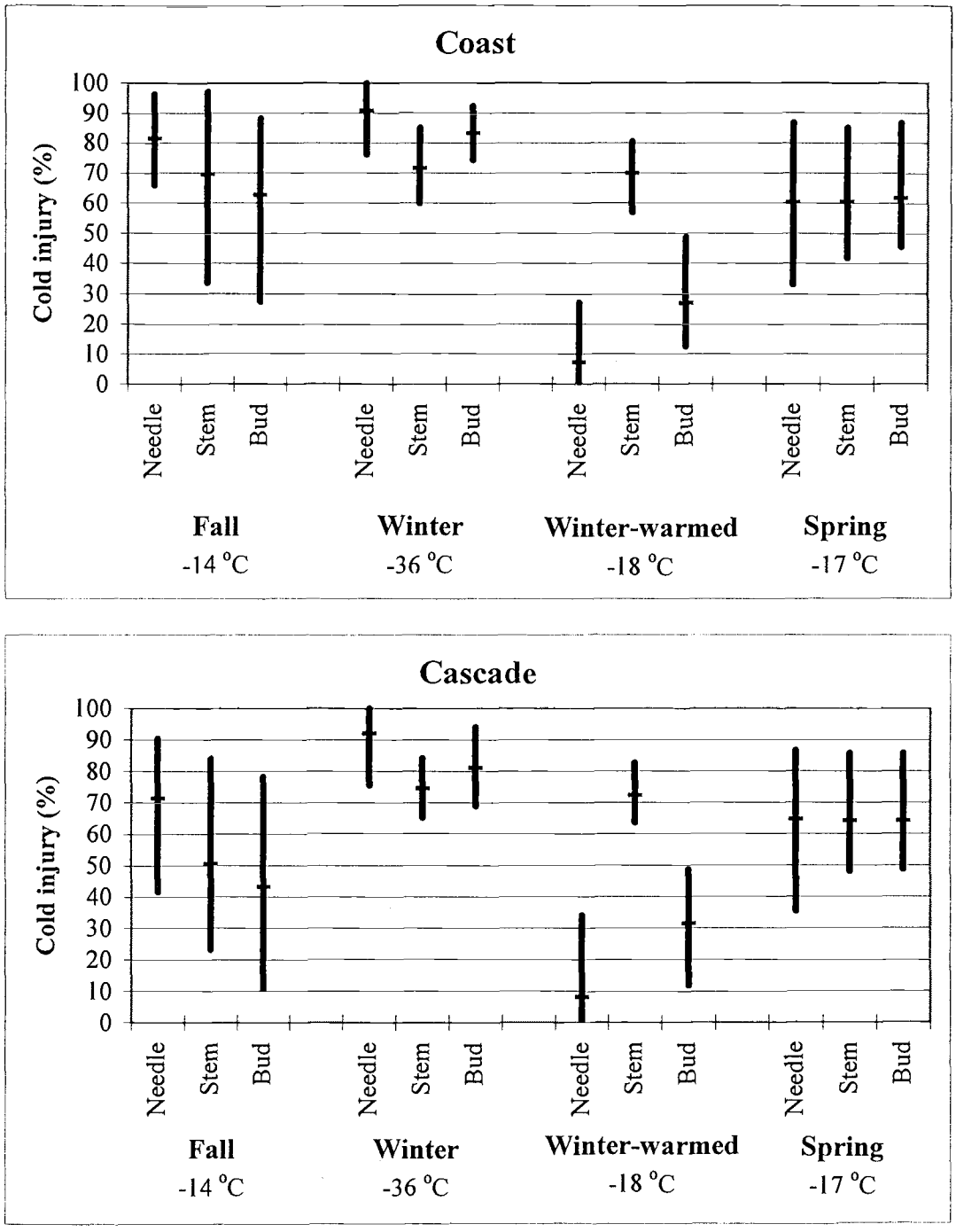

Figure 4.1. Population means (horizontal bars) and family ranges in cold injury (% tissue damaged) to needles, stems and buds following artificial freeze testing of detached shoots in fall, winter, winter after 11 days of artificial warming (winter-warmed), and in spring to seedlings from two western Oregon populations (Coast and Cascade) of Douglas-fir. Mean test temperature is indicated for each treatment. Each population is represented by 40 families. Injury scores are mean scores across the two test temperatures for each treatment.

Future studies of dehardening should therefore examine dehardening rates among tissues and assess the effect of using detached shoots in dehardening experiments.

Needles and buds were substantially hardier in the winter-warmed assessment than in the spring assessment (Fig. 4.1) despite the fact that seedlings in both assessments received similar heat sums. Use of detached shoots in the winter-warmed assessment may also have impacted these results. Whole plant controls were not used in the winter-warmed assessment to examine the effect of detaching on dehardening of the leader. This precludes considering possible effects of developmental or dehardening factors (Fuchigami *et al.* 1971; Timmis and Worrall 1974) translocated from the lower portion of the shoot in the intact seedlings used in the spring assessment (which might explain the greater cold hardiness of needles and buds in the winter-warmed than in the spring assessment). Alternatively, the rapid warming rates used in the winter-warmed assessment (16/20 °C day/night) may have exceeded the maximum dehardening rate for needles and buds. Consequently, needles and buds may have been more cold hardy had the warming occurred more slowly, as in the spring assessment.

Genetic variation and control of cold hardiness

Family variation in cold hardiness tends to be maximum during mid-hardening and mid-to-late-dehardening stages of the annual developmental cycle (Rehfeldt 1979; Aitken and Adams 1996, 1997), and family differences in fall hardiness have been attributed to different dates of onset of hardening (Aitken and Adams 1996). Consistent with this tendency, family variance was greater in the fall (mean $CV_F\% = 18.5$) and spring (mean $CV_F\% = 16.3$) than in the winter (mean $CV_F\% = 6.8$) (Table 4.2). These results are reflected in heritabilities which averaged two and three times the magnitude in the fall ($\overline{h}_i^2 = 0.40$) and spring ($\overline{h}_i^2 = 0.68$), respectively, than in the winter ($\overline{h}_i^2 = 0.22$). Similar results and conclusions regarding family variation in winter and spring cold hardiness were obtained for the same families at age 7 (Aitken and Adams 1996, 1997).

Genetic variation in cold hardiness was also considerably weaker in the winter-warmed assessment (significant differences ($p < 0.05$) among families were detected in only three of six population x tissue combinations) than in the spring assessment (family differences were highly significant ($p < 0.001$) for all tissues in both populations) despite the fact that the average level of cold hardiness was roughly equivalent in the two assessments. The rapid dehardening rate in

Table 4.2. Family variance components expressed as family coefficient of variation (CV_F%), and individual heritability (h_i^2) of cold injury scores following artificial freeze testing of seedlings (detached shoots) from two western Oregon breeding populations (Coast and Cascade) of Douglas-fir in fall (October), winter (February), spring (March), and in winter after a brief period of warming.

		Fall		Winter		Winter-warmed		Spring	
Zone	**Tissue**	**CV_F%**[ab]	**h_i^2**[c]	**CV_F%**	**h_i^2**	**CV_F%**	**h_i^2**	**CV_F%**	**h_i^2**
Coast	**Needle**	6.6 ***	0.19	10.0 **	0.28	---[d]	---	19.6 ***	0.67
Cascade		11.5 ***	0.32	8.6 *	0.25	44.0[e] *	0.17	14.4 ***	0.52
Coast	**Stem**	20.3 ***	0.66	5.5 *	0.19	4.3 *	0.21	19.3 ***	0.83
Cascade		22.0 ***	0.35	7.0 *	0.19	4.0 **	0.24	13.5 ***	0.55
Coast	**Bud**	21.5 ***	0.47	---	---	---	---	16.8 ***	0.78
Cascade		28.9 ***	0.43	3.1 *	0.17	---	---	13.8 ***	0.70

[a] Forty families used to calculate CV_F%.

[b] Significance of family variance: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

[c] Average standard error of individual heritabilities = 0.14 (range 0.12 - 0.16).

[d] Value not estimated due to non-significant family variance.

[e] This anomalous value is likely an artifact of the low level of injury achieved in this assessment.

the winter-warmed treatment likely compressed the range in phenological stages among families, reducing the family variation in cold injury in this assessment. Furthermore, the degree and duration of the dehardening treatment were selected to ensure that a moderate level of dehardening would occur, so that the probability of detecting an effect of the mid-winter warming treatment would be maximized. However, the probability of experiencing in western Oregon, a mid-winter warm event of the magnitude (T_m = 18 °C) and extent (11 days) employed in this experiment is small. Therefore, in future dehardening studies it may be prudent to select dehardening rates and durations most likely to occur, rather than those most likely to produce an effect.

The limited family variation in cold hardiness in the winter-warmed assessment relative to the spring assessment may be related to the phenomenon of re-hardening - i.e., re-acclimation following partial dehardening, which has been reported in nature (Strimbeck *et al.* 1995) and under artificial conditions (Repo 1991; Leinonen *et al.* 1997). Re-hardening was observed in *Pinus sylvestris* clones in Sweden upon a return to seasonal temperatures which followed a period of unusually warm winter weather. However, not all clones re-hardened (Nilsson and Walfridsson 1995). In the current study, if some families re-hardened more than others during cold weather in the late winter (freezing temperatures were recorded on seven days between February 1 when the warming treatment began, and March 24 when spring AFTs were conducted), family variation in cold hardiness may have increased in the spring assessment. Consistently warm temperatures in the winter-warmed assessment would have precluded re-hardening of any families in this assessment. Bud burst/dehardening models (Campbell and Sugano 1979; Cannell and Smith 1986; Cannell 1989b; Hänninen 1990; Hannerz 1999) may therefore benefit from an examination of the role of re-hardening in spring phenology.

Genetic correlations between tissues

Estimated genetic correlations between tissues in cold injury were positive and generally strong in both fall and spring in seedlings (Table 4.3 and see Chapter 2). However, correlations between tissues in winter and in winter after warming were weaker and highly variable, ranging from -0.70 to 0.74. Weak genetic correlations in cold injury between tissues in winter were also observed in saplings of these families (Aitken and Adams 1996). Weak family variation for cold hardiness traits in the winter and winter-warmed assessments likely contributes to the weak genetic correlations between tissues in these assessments. Also, use of fewer seedlings per

Table 4.3. Estimated genetic correlations between needle, stem and bud tissue cold injury scores in fall, winter, spring, and in winter after warming, in seedlings from two western Oregon populations (Coast and Cascade) of Douglas-fir.

Correlation	Fall [a]		Winter		Winter-warmed		Spring [a]	
	Coast	Cascade	Coast	Cascade	Coast	Cascade	Coast	Cascade
needle-stem	0.97	0.67	0.74	0.67	---[b]	0.27	0.78	0.92
needle-bud	0.68	0.47	---	-0.42	---	---	0.75	0.89
stem-bud	0.95	0.77	---	-0.70	---	---	0.95	0.93

[a] Estimates based on seedlings from wet moisture regime only.

[b] Genetic correlation not calculated when family variance not significant.

family in the winter and winter-warmed assessments (8 seedlings), relative to the fall and spring assessments (16 seedlings) contributes to the more variable estimates in the winter and winter-warmed assessments.

Relationships between winter cold hardiness after warming and other traits

In the Coast population, it appears that moderately strong positive genetic correlations exist between cold injury in the winter-warmed and winter assessments ($r_A = 0.68$), and between cold injury in the winter-warmed and spring assessments ($r_B = 0.75$) in stems. However, as a result of weak genetic variation and the use of only 8 observations in the winter-warmed assessment, substantial error is expected to be associated with these estimates (Table 4.4). Nonetheless, these results suggest a moderate degree of overlap between genes regulating winter or spring cold hardiness and genes regulating cold hardiness following winter warming of stems in the Coast population. Consequently, selection for either winter or spring hardiness should result in greater stem cold hardiness following winter warming. In contrast, in the Cascade population, stem cold hardiness following mid-winter warming was found to be essentially unrelated to winter or early spring cold hardiness (r_A = -0.12 and 0.15, respectively).

Re-hardening may also help to explain differences between populations in family stability ranking during dehardening. If dehardening and re-hardening are under different genetic control, freezing nights may have re-hardened seedlings in the winter and spring assessments, altering family rankings, relative to rankings in the winter-warmed assessment. Higher threshold temperatures on the coast result in slower dehardening in Coast populations, where rate of change of minimum daily temperature during early spring is more gradual than in the Cascades. Consequently, if re-hardening does occur during cold periods in early spring, it may be less prevalent in Coast populations, where warming would deharden trees less than in Cascade populations. Therefore, a greater frequency of re-hardening in the Cascade population may account for the weaker correlations of cold injury in the winter-warmed assessment with winter and spring injury.

Conclusions

Narrow family variation and weak heritabilities for cold hardiness following a brief period of mid-winter warming imply that 1) populations of Douglas-fir in western Oregon may have limited potential to adapt to natural mid-winter warming events; 2) gain through artificial

Table 4.4. Genetic correlations of cold injury scores from artificial freeze tests in winter (February 1997) after a brief period of warming, with cold injury scores in fall (October 1996), winter (February 1997) and spring (March 1997) in seedlings from two western Oregon populations (Coast and Cascade) of coastal Douglas-fir.

Correlation	Needles		Stems		Buds	
	Coast	Cascade	Coast	Cascade	Coast	Cascade
winter-warmed - fall	---[a]	-0.10	-0.30	-0.79	---	---
winter-warmed - winter	---	0.46	0.68	-0.12	---	---
winter-warmed - spring	---	0.65	0.75	0.15	---	---

[a] Genetic correlation not calculated when family variance not significant.

selection for cold hardiness following mid-winter warming will be slow; and 3) selection for other cold hardiness traits will have limited impact on cold hardiness following mid-winter warming. These conclusions, however, are preliminary. Results in this study will require confirmation with larger samples sizes and with a range of dehardening treatments. Furthermore, the effect of using detached shoots in dehardening experiments and the role of re-hardening warrant evaluation.

Chapter 5

General Conclusions

Conclusions

Results of these investigations demonstrate that artificial freeze testing (AFT) provides reliable estimates of family cold hardiness values that are cheap and easy to obtain for large numbers of individuals. The reproducibility of family rankings for cold injury across test dates, tissues, soil moisture conditions and tree ages speaks not only to the similarity of the genetic control of cold hardiness in different tissues and environments, and at different ages, but also to the accuracy of the AFT procedure. Advances in the understanding of the genetics of cold hardiness in Douglas-fir, together with the refinement of screening procedures achieved with these and previous PNWTIRC studies, can facilitate identification of cold hardy families for deployment in reforestation, and for selection in breeding programs.

Conclusions to this thesis may be summarized as follows:

- Considerable genetic differentiation exists between the Coast and Cascade populations for fall cold hardiness at the seedling stage. Differentiation between populations was much less for spring cold hardiness.
- Fall and spring cold hardiness in seedlings varied widely among families of coastal Douglas-fir in two western Oregon breeding zones (Coast and Cascade) and are under strong genetic control ($\overline{h}_i^2$ = 0.57 in March and 0.37 in October).
- Family rankings for fall and spring cold hardiness are fairly consistent across different summer soil moisture regimes, different tissues and different fall dates.
- Family cold hardiness in fall and spring were negatively associated in the Coast population, but nearly uncorrelated in the Cascade population.
- Family rankings for fall cold hardiness were fairly consistent between artificial freeze tests and a natural frost.
- Cold hardiness in seedlings and saplings is under similar genetic control.

- Bud burst timing is strongly associated with spring cold hardiness in seedlings and saplings. Bud set timing is strongly associated with fall cold hardiness in seedlings, but not in saplings.
- Family variation and heritabilities are weak for cold hardiness in winter ($\overline{h}_i^2 = 0.22$) and in winter after a brief period of warming ($\overline{h}_i^2 = 0.21$).
- Genetic relationships of cold hardiness in winter after a brief period of warming, with winter and spring cold hardiness were fairly strong and positive in the Coast population, but were weak and inconsistent in the Cascade population.

The success of this experiment may be credited to good experimental design (control of genetic materials, use of a large number of families, randomization, replication, etc.), and attention to cultural techniques (uniform nursery conditions, good germination, effective moisture regime application, etc.) and freeze test procedures (effective test temperature selection procedure, timely and consistent sampling routine, consistent injury scoring system, etc.). The strength of the results was enhanced by being able to corroborate seedling results with results from sapling experiments. However, use of additional breeding populations derived from more contrasting environments may have enabled stronger conclusions to be drawn regarding population differences. Also, if the environmental variation of parent tree sites within a population was similar among populations, associations could have been made between genetic and environmental parameters. Finally, estimating the genetic association between cold hardiness and growth traits was not performed because stem growth was complicated by the natural frost event which affected seedling height. Frost injury prevention measures may have reduced the impact of the frost on height growth and permitted evaluation of the association of cold hardiness and height growth.

Future research

The precision of cold injury scores using the AFT procedure appears remarkable, as indicated by individual heritabilities for spring cold hardiness which often exceed 0.60. However, further refinement of the procedure may be desired for cold hardiness traits having lower heritabilities, or if relationships between cold hardiness in AFTs and field performance following cold stress show that significant differences in important field performance traits occur within a small range of AFT scores.

Different interpretations of cold injury among personnel scoring the injury is expected to be a main cause of variation within and among tests. Minimizing the number of personnel used to score the injury and 'blocking' by personnel will help to control this source of variation. In addition, the use of color photographs of tissues at a range of injury levels as a reference for scoring personnel may help to standardize scoring schemes and reduce error variance. Also, cold hardiness appears to vary considerably with branch size and position within the seedling. This source of variability may be reduced if testing is done when seedlings are older and the availability of branches of similar size and from the same position within the seedling is greater.

AFT freezing rates are generally 3-5 °C/h, but faster rates have been reported in nature (van der Kamp and Worrall 1990). Because the rate of freezing may influence the effectiveness of freezing mechanisms (Sakai 1982), it may be worthwhile examining the extent to which different families respond to different freezing rates in AFTs.

Strong genetic correlations between injury in AFTs and following a natural frost event in seedlings (Chapter 2), saplings (Aitken and Adams 1997) and in other studies (Rehfeldt 1986; Nilsson and Walfridsson 1995) suggest that relative cold hardiness determined in AFTs is a fairly good predictor of relative frost hardiness in field conditions. However, to reliably predict the performance of a genotype under cold stress in the field (i.e., its field cold hardiness), methods developed in these studies will have to be extended to relate cold hardiness in AFTs to growth, form and survival following cold stress in field studies. Relating cold hardiness of detached shoots in AFTs to whole plant survival and growth in AFTs (Keates 1990) is one approach the evaluating the implications of AFT rankings. Alternatively, relating cold hardiness in AFTs to growth, form and survival following cold stress in the field may be achieved using uniform, agricultural-like 'farm-field' sites (Woods *et al.* 1995) situated in frost prone locations. This strategy, and the controlled application of damaging frost using mobile freezers and tents to contain the frost over a farm-field site, may help to mitigate problems of site non-uniformity and infrequency of discriminating natural frosts (i.e., frosts capable of distinguishing among families in level of cold hardiness) associated with field tests of cold hardiness.

Once reliable relationships between cold hardiness in AFTs, and growth, form and survival in field tests following cold stress are established, the performance of a subset of reference families across a range of expected cold hardiness levels could be easily determined. The AFT

ranking of test families relative to the reference families could then be used to predict field performance of the test families using AFTs. Having predicted the relative cold hardiness of genotypes in the field, cold injury risk models are then required in order to efficiently deploy the genetically improved materials (i.e., families or clones) in different cold risk areas. Refinement of cold injury risk models (Timmis *et al.* 1994) and their development at a fine scale would greatly help in this regard.

Bud burst date can be used to effectively rank families for spring cold hardiness. However, neither bud burst date alone, nor AFTs conducted at only one or two test temperatures, allow estimation of absolute values of cold hardiness (e.g., LT_{50} or T_c - critical temperature, DeHayes and Williams, 1990) throughout the dehardening period. The ability to predict absolute cold hardiness (i.e., not merely relative cold hardiness ranking) throughout the dehardening period will be required in order to estimate cold injury risk, which may be necessary in the event of global warming, or if seed are to be transferred to different environments.

The risk of winter or early spring cold injury may be estimated from winter weather records if the absolute value of cold hardiness (e.g., LT_{50}) is known on each date during dehardening. The absolute value of cold hardiness on any date during dehardening can be estimated from the relationship between LT_{50} and HS_n / HS_{req}. The heat sum expected on a given spring date (i.e., HS_n) can be calculated from the threshold temperature (T_{th}) and HS_{req}, which can be estimated by growing genotypes from different climatic regions in a common garden experiment under contrasting spring temperature regimes (Worrall 1983). Experiments such as these will help to ensure that deployed seedlings will thrive in their planted environments, and will also serve as a model for assessing genetic variation in other adaptive traits.

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