

Cold Hardiness Testing for Douglas-Fir Tree Improvement Programs: Guidelines for a Simple, Robust, and Inexpensive Screening Method

T.S. Anekonda, W.T. Adams, *Department of Forest Science, 321 Richardson Hall, Oregon State University, Corvallis, OR 97331-5752*, and S.N. Aitken, *Department of Forest Sciences, University of British Columbia, 3041-2424 Main Mall, Vancouver, BC V6T 1Z4*.

ABSTRACT: Operational methods are needed for screening genotypes in breeding programs for adaptive traits. In this article, we present a detailed description of one procedure for screening improved coastal Douglas-fir seedlings and saplings for cold hardiness, based on research results of the Pacific Northwest Tree Improvement Research Cooperative. Artificial freeze testing of detached shoots from genetic tests, followed by visual scoring of injury, has proved to be an efficient, reliable, and cost-effective method of screening large numbers of genotypes. Relevant research results are summarized, and practical details of this methodology are presented for straightforward implementation by Douglas-fir breeders and researchers. *West. J. Appl. For.* 15(3):129–136.

Although losses in coastal Douglas-fir (*Pseudotsuga menziesii* var *menziesii*) from cold damage are rarely reported in the literature (Munger and Morris 1936, Duffield 1956, Griffith 1960, Van Haverbeke 1987), let alone assessed in terms of economic impact, foresters in the Pacific Northwest are well familiar with injury that occurs in young plantations due to sporadic cold events. In “frost-prone” sites, damage occurs more frequently than on milder sites and can be severe, leading to mortality, reduced growth, and poor stem form. The greatest risk of cold injury occurs in the Fall (Oct.–Nov.) before shoots are fully hardened for the winter or in the Spring (mid-April to mid-May) when shoots begin, or are about to begin, active growth. Timmis et al. (1994) quantified frost damage risk for Douglas-fir in Oregon and Washington west of the crest of the Cascade Mountains using climate models and weather records in combination with seedling cold hardiness data. Although the most severe frosts in this region are likely to occur in the spring, damaging fall frosts are expected more frequently than damaging spring frosts at about 30% of the weather stations included in the study. Thus, the potential for both spring and fall frost injury needs to be considered in cold hardiness assessments.

Considerable genetic variation in both spring and fall cold hardiness has been observed within breeding populations of

coastal Douglas-fir (Silen 1978, Loopstra and Adams 1989, Wheeler et al. 1990, Aitken et al. 1996, Aitken and Adams 1995a, 1996, 1997, O’Neill et al. 1996, Anekonda et al. 1998). This variation can be exploited in tree improvement programs in two ways: (1) in selection and breeding to maintain or enhance levels of cold hardiness of improved strains; and/or (2) in choosing specific families to plant in frost-prone sites. To be employed in practical tree improvement programs where large numbers of individuals must be assessed, cold hardiness screening techniques need to be cost-efficient, as well as effective. In this article, we describe the cold hardiness testing procedure we use for genetics research in the Pacific Northwest Tree Improvement Research Cooperative and offer suggestions for its application in routine screening for tree improvement.

Methods of Assessing Cold Hardiness in Trees

Cold hardiness can be assessed after damaging frost events in the field or after artificial freezing of whole seedlings or cut stems. We also address the utility of scoring shoot phenology as a means of indirectly assessing hardiness of stems.

Field Assessment of Cold Injury

Assessing progeny tests in the field for injury following natural frosts provides a direct measure of sensitivity to actual cold events and implications of cold injury for stem form and growth. Incidence of natural frost injury, however, is typically sporadic across field test sites and over

NOTE: Thimmappa S. Anekonda is the corresponding author and can be reached at (541) 737-6579; Fax: (541) 737-1393; E-mail: Thimmappa.Anekonda@orst.edu. This is Paper 3354, Forest Research Laboratory, Oregon State University.

time, leading to uneven testing and poor statistical precision. In addition, it is difficult to separate frost damage from other causes of injury (e.g., drought, disease, insects). Because of these difficulties, we and others have turned to assessing injury after artificial freeze testing, where the temperature of freezing can be strictly controlled and uniformly applied.

Artificial Freeze Testing

One problem with artificial freeze testing is that the relationship between freeze-injury scores and cold hardiness under actual field conditions (i.e., extent of injury, survival, impact on growth and stem form) is not clear. Without results from experiments that relate field performance to test results, this will always be true. Two recent studies in coastal Douglas-fir, however, found moderate to strong estimated genetic correlations between visual stem injury scores following artificial freezing and natural frost events (mean = 0.82, range = 0.47–1.00), suggesting that artificial freeze testing can provide reliable predictions of the relative hardiness of families to both spring (Aitken and Adams 1997) and fall frosts (O'Neill 1999).

Materials

Artificial freeze testing is done on either whole seedlings or on detached shoots.

Whole Seedlings.—When whole seedlings are used in freeze testing, implications of cold injury for whole plant survival, growth and stem form can be assessed (Timmis 1976, Ritchie 1984, Rietveld and Tinus 1987). Disadvantages of this method are that only seedlings (and not larger trees) can be tested, only a small number of individuals can be sampled at a time, and testing typically results in destruction of the whole plant.

Detached Shoots.—There are no restrictions on the age of trees that can be sampled when detached shoots are used for cold injury assessment. In addition, large numbers of samples can be tested efficiently (Warrington and Rook 1980, Aitken et al. 1996). A major disadvantage in comparison to seedling testing is that only the relative degree of cold hardiness can be assessed; that is, the meaning of cold injury scores in terms of tree survival, growth, and stem form is not entirely clear.

Assessing Injury

Cold injury can be assessed quantitatively by measuring electrolytic conductivity and chlorophyll fluorescence of freeze-tested tissues or subjectively by visual scoring. Use of electrolytic conductivity and chlorophyll fluorescence is described and compared in the literature (Burr et al. 1990, Glerum 1985, Warrington and Rook 1980). Here we present a simple method for assessing cold hardiness using visual injury, a reliable technique requiring little equipment or technical training.

Visual assessment is quick, and large numbers of samples can be efficiently processed at one time (Aitken et al. 1996, Aitken and Adams 1996 and 1997). With visual assessment, frozen tissue is allowed to develop damage symptoms for several days after freezing and then scored into damage classes. Damage is assessed with less precision than with electrolytic conductivity and chlorophyll fluorescence and is

subject to further error depending on the skill and experience of the scorer. Nevertheless, injury assessment is simple, straightforward, and requires little technical knowledge. In addition, damage to all three stem tissues (i.e., buds, stems [phloem and xylem], and needles) can be evaluated simultaneously.

Visual damage scores have been found to be strongly correlated ($r^2 > 0.90$) with injury assessed quantitatively using electrolytic conductivity (Shortt et al. 1996) and chlorophyll fluorescence (Binder et al. 1997). Further evidence of the reliability of visual scoring is the strong (and statistically significant) family differences we generally find for injury scores (Aitken et al. 1996, Aitken and Adams 1996, 1997, also see below). Because visual scoring has proven to be reliable and requires little specialized equipment or training, we recommend this procedure for screening cold hardiness of large numbers of individuals in selection programs.

Expression of Cold Hardiness

Cold hardiness is usually expressed either as the temperature that inflicts 50% tissue damage (LT_{50}) or just simply as percent damage at one or more temperatures. To estimate LT_{50} , tissue must be tested across a range of freezing temperatures that inflict low to high levels of injury, and the temperature that would result in 50% damage interpolated by plotting or regression techniques (Burr et al. 1990, Sakai and Larcher 1987). Estimation of LT_{50} is quite laborious because it requires testing at several temperatures (four or more are recommended) in order to ensure accurate interpolation of the 50% damage point. We prefer the simpler method of freezing at temperatures that inflict intermediate levels of damage (i.e., 30%–70%) and then ranking families on the basis of their percent damage score. The difficulty here is making sure that the temperatures chosen will, indeed, inflict intermediate levels of damage. To ensure this, we perform a preliminary test on a few individuals (discussed later); and then to be safe, we use two temperatures 2°–3°C apart for testing. With two temperatures, there is a good probability that at least one will give the intermediate damage levels required. When both test temperatures give intermediate damage levels, we have found that genetic correlations between injury scores at the two temperatures are very high (Aitken and Adams 1996, 1997). Thus, precision of cold injury assessment can be increased by using the mean of two injury scores. In addition to being easier and less costly to determine, we found that percent damage scores are highly correlated with LT_{50} values. For example, mean LT_{50} 's (based on four test temperatures) and percent damage (two temperatures) were estimated for 180 clones of individuals (two to four ramets each) from a single full-sib family of coastal Douglas-fir. Correlations between the two measures of cold injury ranged from 0.82 to 0.94 across three tissue types (Figure 1).

Indirect Assessment of Shoot Cold Hardiness by Scoring Bud Phenology

Stem dehardening and hardening are intimately linked to the timing of budburst and budset, respectively. Indeed,

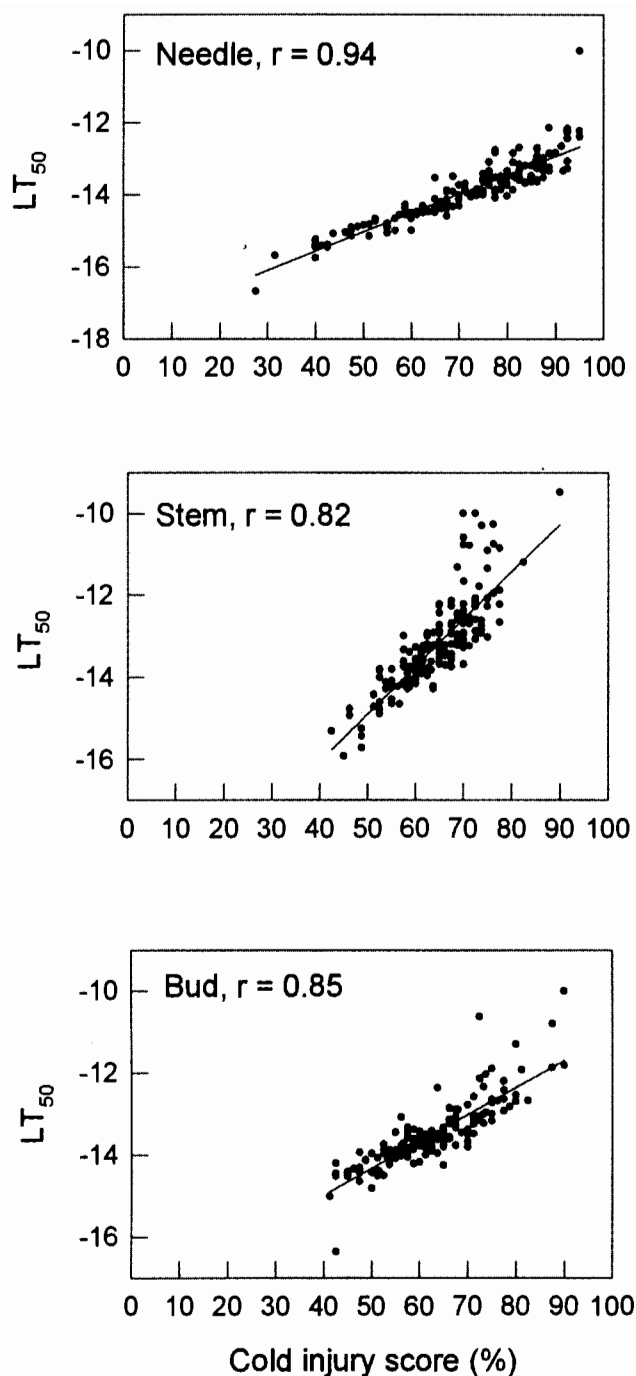


Figure 1. Regression of spring cold injury LT_{50} values (estimated using four test temperatures) on cold injury scores (mean percent damage for two intermediate freezing temperatures for three shoot tissues (needle, stem, bud)) of 180 Douglas-fir clones. Clones were individuals (2–4 copies of each) in a single full-sib family from a western Oregon breeding program.

dehardening of stems in late winter and early spring culminates in budburst and growth of new shoots. Budset and initiation of stem hardening are triggered by shortening daylength, although the timing of both may be advanced by summer drought (Weiser 1970, White 1987). Thus, we would expect late-flushing genotypes to be the hardiest when exposed to a spring frost and those that set bud earliest to be the hardiest to fall frost. These expectations are generally met in coastal Douglas-fir (Munger and Morris 1936, Loopstra and Adams 1989, Aitken et al.

1996, Aitken and Adams 1996, 1997, O'Neill et al. 1996). Nevertheless, while genetic correlations between date of budburst and stem cold injury are consistently strong in both seedlings and saplings (mean $r_g = -0.88$, range = -0.82 to -0.92), genetic correlations between timing of budset and fall cold hardiness are small in saplings (mean $r_g = 0.33$, range = 0.28 – 0.38), but are moderate to strong in seedlings (mean $r_g = 0.80$, range = 0.65 – 0.96) (Aitken and Adams 1995a, Aitken et al. 1996, 1997, O'Neill 1999). Thus, it appears that family ranking for spring cold hardiness can be reliably assigned on the basis of budburst timing in both seedlings and saplings, but budset timing may only reliably be used as a substitute for fall cold hardiness ranking in seedlings.

The advantage of using bud phenology as a surrogate for spring or fall cold hardiness is that no specialized equipment or collection of samples is required. Nevertheless, accurately estimating bud phenology can be tricky, especially if it is done on trees in the field. The preferred method of scoring bud phenology is to visit tests once or twice weekly during budburst and budset periods, noting when the terminal bud (of leader or lateral shoots) of individual trees has flushed (budburst) or when bud scales are first visible on the apical meristem (budset) (Campbell and Sorensen 1973, Campbell and Sugano 1975, 1979, Li and Adams 1993). This might entail a half dozen or so visits to tests in each season in order to cover the range of budburst or budset dates of all individuals in the population. An alternative that works well for ranking families for relative budburst timing is to visit the test once midway in the budburst period and tally the proportion of trees per family that have flushed (Li and Adams 1993). This proportion has a strong negative genetic correlation ($r_A \leq -0.93$) with mean budburst date, but requires that the populations be assessed when between 25% and 75% of all trees in the test have flushed. It can be quite difficult to determine when the proper stage of flushing has been reached in a particular test because timing can vary by several weeks depending on weather. This is less of a problem in a nursery test, which may be more convenient to visit on a regular basis. Scoring budset, even in a nursery, is more difficult because the apical meristem is hidden by new needles. In addition, the presence of second flushing in young Douglas-fir (see next section) requires that budset continue to be scored on a weekly basis until re-flushing of shoots no longer occurs. Tests need to be visited only once in the spring and in the fall to collect samples for freeze testing, and this can occur anytime within a period of 4–8 wk in both seasons (see below).

Because cold hardiness can be assessed more accurately when cold injury is measured directly and perhaps less expensively than scoring for bud phenology, we feel that artificial freeze testing is the preferred method for ranking Douglas-fir genotypes or families for cold hardiness. Scoring phenology is a suitable substitute for ranking cold hardiness in seedlings only when the required time and effort can be applied to precisely estimating date of budburst and final date of budset.

Genetics of Cold Hardiness in Coastal Douglas-Fir

The Pacific Northwest Tree Improvement Research Cooperative has studied the quantitative genetics of spring and fall cold hardiness using materials from 291 families in a total of five breeding populations from British Columbia (1), Oregon (2), and Washington (2) (Aitken and Adams 1995a, 1995b, 1996, 1997, Aitken et al. 1996, O'Neill 1999, Anekonda et al. 1998). In all cases, we used artificial freeze testing of cut shoots and visual scoring of damage. Fall cold hardiness was examined in sapling-age trees (4–7 yr) growing in field sites in both Washington populations and in the British Columbia population, while both fall and spring cold hardiness were evaluated at both the seedling and sapling stages in the remaining two (Oregon) populations. Because cold hardiness appears to be controlled largely by the same set of genes in the two age classes, as indicated by high genetic correlations and thus consistent family rankings between the two ages (O'Neill 1999), we restrict our summary to results from the sapling studies.

Variation Among Tissues

When assessing artificial freeze damage of shoot samples, a number of choices can be made on what tissue or tissues to score. Internal injury to stems (i.e., cambium and phloem) and buds likely has the greatest impact on future stem growth and stem form, but needle injury is easier to observe (i.e., requires no cutting to observe damage). In the spring, strong genetic correlations in freeze damage are found among the three tissue types (mean $r_g = 0.88$, range = 0.74 to 0.98), indicating scoring one tissue is adequate for ranking families for cold hardiness of all tissues (Aitken and Adams 1997). In the fall, the relationship between tissues for cold hardiness rating is less consistent. In late fall (November), the temperatures required to achieve intermediate damage levels in needles and stems inflict severe damage to buds (i.e., killing most or all buds), such that it is not possible to use the same test temperatures for all three tissues (Aitken and Adams 1996). All these tissues can be reliably scored with the same freeze temperatures in early fall (September or October), but genetic correlations in injury between tissues are generally not strong (mean $r_g = 0.61$, range = 0.16–0.92, Aitken et al. 1996, Aitken and Adams 1996). Because of the importance of stem injury, we recommend that this tissue, at least, be scored in both fall and spring. In the fall, scoring needle or bud tissues may not give reliable ranking of stem cold hardiness. In the spring, it is more effective to score stem cold hardiness directly because the heritability of stem injury appears to be about 50% greater than in needles or buds (Aitken and Adams 1997).

Budsetting and reflushing of shoots one or more times before final budset (called "second flushing") is a common phenomenon in Douglas-fir seedlings and young trees (up to ~ 10 yr) growing on favorable sites (Adams and Bastien 1994). When assessing stems for fall cold hardiness, it is necessary to be consistent in sampling shoots from all individuals that have either all second flushed or all not second-flushed. This is because second flushed shoots have consid-

erably less cold hardiness in early fall than shoots that flushed only once (Anekonda et al. 1998). We suggest using single-flush shoots, when possible, because these shoots are more relevant to shoot cold hardiness at later ages when second flushing ceases in Douglas-fir.

Genetic Control

Because, on average, freeze injury is moderately to strongly correlated between tissues in fall and spring, we refer only to stem cold hardiness in the remainder of this section. In all our studies, family variation in cold injury scores has been extensive, often ranging from less than 10% mean damage of stems to 40% or more damage among the 40–89 families in each population sample (Aitken et al. 1996, Aitken and Adams 1996, 1997). Estimated individual-tree heritabilities of cold injury were much higher in the spring (mean $h^2 = 0.72$) than in the fall (mean $h^2 = 0.20$) in the two Oregon populations where direct comparisons could be made (Aitken and Adams 1996, 1997). Heritabilities for fall cold injury, however, are still large enough that it is possible to make good progress in genetically improving fall cold hardiness by selection and breeding. In both spring and fall, estimated genetic correlations between cold injury scores made on different sampling dates in the same year, different sites on the same sampling date, between similar sampling dates in different years, and between different age classes (seedlings versus saplings) were high, averaging more than 0.85 in each category (Aitken and Adams 1996, 1997, Aitken et al. 1996, O'Neill 1999). This indicates that there is little genotype \times environment (or age) interaction in cold injury scores and that a single assessment at one age at a single test site should be sufficient for ranking families for cold hardiness in each season. Genetic correlations between fall and spring cold injury, however, were weak (mean $r_g = -0.25$, range = -0.36 to -0.14), indicating that cold hardiness in the two seasons is largely under the control of different sets of genes, and that fall and spring cold hardiness must be assessed separately (Aitken and Adams 1995a, O'Neill 1999).

Genetic Relationships Between Cold Hardiness and Stem Growth

It is important to understand the implications of ignoring cold hardiness when emphasizing selection for faster height growth. We investigated the relationship between spring cold injury and tree height in the two Oregon breeding populations and found a moderate, positive correlation in one population and a weak, negative correlation in the other (Aitken and Adams 1995a). We found estimated genetic correlations between height growth and fall cold injury to stems to be weak ($-0.22 \leq r_A \leq 0.21$) in all four breeding populations (two in Oregon and two in Washington) for which we have investigated this relationship (Aitken and Adams 1995a, 1996). There was little second flushing, however, in the years we assessed these tests. Because second flushing increases susceptibility to injury from fall frost (Anekonda et al. 1998) and because trees with a propensity to second flush have faster height growth on favorable sites (Adams and Bastien 1994, Schermann et al. 1997), we expect that growth and fall cold hardiness will be unfavorably correlated on sites where

trees are prone to second flushing. Given that correlations between height growth and cold hardiness are neither consistent nor clear, the potential impact of selection for height growth on cold hardiness should be evaluated on a case by case basis.

Cold Hardiness Testing Procedure

Our cold hardiness testing procedure involves three steps: (1) preliminary test, (2) main freeze test, and (3) data analysis. We will discuss the main test first since the freeze-testing protocol in the preliminary test is basically the same.

Main Freeze Test

Collection of Samples

Cut one 4–6 cm long lateral shoot tip from lateral branches of each tree being investigated for each test temperature. Shoots should be healthy (no damage evident) and have well-developed terminal buds. Three essential components of sample collection for cold hardiness testing are: (1) experimental error must be minimized, (2) shoots must be handled with care, and (3) identity of shoot samples must be maintained throughout.

- 1. Minimize Experimental Error.** Sampling must be done according to the experimental design of the test; i.e., collect cuttings by block and keep them together by block throughout processing. If all samples from a test cannot be collected in 1 day, it is important to make sure that individual blocks are completely sampled (i.e., don't collect from some trees in a block on one day and from the remainder on another day). In this way, any differences resulting from day of sampling can be combined with block effects in the analysis and will not affect the ranking of individual families. Based on a limited investigation of the effects of shoot location in the crown (i.e., height and aspect) on cold hardiness (Pacific Northwest Tree Improvement Research Cooperative, 1992–93 Annual Report, Forest Research Laboratory, Oregon State University), we have adopted the following criteria for sampling shoots to further minimize sampling error: (1) All shoots must come from branches fully exposed to sunlight in the mid to upper crown. (2) Given that (1) is met, shoots be sampled as uniformly as possible with regards to height above the ground. (3) Samples should come from the same flushing type (i.e., all second-flushed or all non-second-flushed shoots, not a mixture of both) and preferably be from the same crown aspect and order of shoot. In older trees, branches at the same height off the ground (criteria 2) may differ somewhat in developmental stage among trees of different total height. Nevertheless, by sampling at the same height off ground, all shoots will come from branches exposed to approximately the same air-temperature regime.

- 2. Handle Shoots with Care.** Once shoots are harvested and labeled they must be kept cool and moist. We place the shoots in plastic bags along with a moist paper towel. Once each bag is full (up to 40–50 samples), it is stored on ice in a cooler until transported to the laboratory.

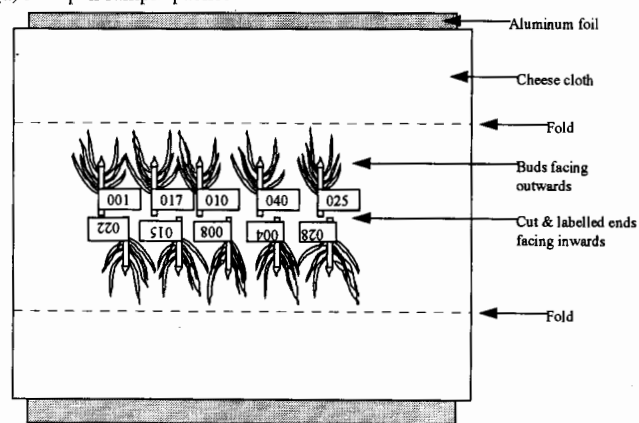
- 3. Maintain Identity.** Each shoot sampled from the same tree is labeled with the same identifying number by wrapping a numbered tape around the base of the detached shoot (Figure 2). For convenience, we custom-order sequentially numbered label tapes. For example, in a test with 1,000 trees and two shoot samples/tree, label tapes with 1,000 consecutive numbers, each repeated twice, are needed. One supplier of sequentially numbered tape is TimeMed Labeling Systems, Inc. (144 Tower Drive, Burr Ridge, Illinois 60521). After labeling all shoots from a given tree, each shoot is placed in a separate plastic bag, which is used to hold samples from up to 50 trees destined for a single freezing-test temperature. The bag is identified by block and test temperature.

Data sheets should be prepared in advance and follow the layout of the test, allowing tree identities (e.g., block, family, row, and column position) to be verified as the samples are collected. It is also convenient to use the same data sheets to record freeze injury scores. Field data recorders can also be used.

Sample Preparation

Samples need to be stored in a refrigerator or cold room (2°C) until freeze testing, which should be conducted as soon as possible following sample collection, ideally within 48 hr. Storage for periods up to 4–5 days, however, is not expected to significantly influence hardiness of samples (De Hayes et al. 1990). Samples are prepared for freezing the afternoon or evening before the freeze run. In one day's run, we usually test one target temperature. We can handle up to 700 samples in a run with our freezer (inside dimensions: 0.47m width; 1.70m length, 0.71m height). It is imperative that, if all samples to be tested at one target temperature cannot be run in one day, all samples from any particular block be processed at the same time.

(a) An open sample packet



(b) A folded sample packet

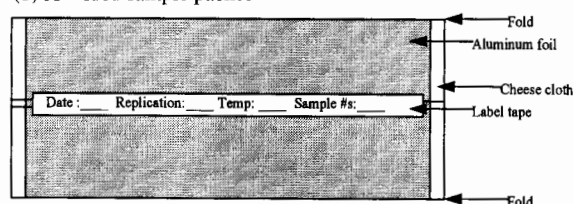


Figure 2. Labeled shoot samples arranged end to end on cheesecloth in preparation of a sample packet for freezing.

All samples in one plastic bag from the field are processed together in the same sample packet. To prepare a sample packet:

1. Cut a piece of grade 50 or 60 cheese cloth to 92 cm × 46 cm and fold over to make a double-layered 46 cm square piece. Soak the cheese cloth in tap water, squeeze moderately to remove excess water, then lay out on to an 46 cm square piece of aluminum foil.
2. Remove all samples from one plastic bag and place on the cheese cloth, arranged in two rows with cut ends (and labels) facing inward (terminal buds outwards) (Figure 2).
3. Fold the cheese cloth, then the aluminum foil, lengthwise over the samples so as not to disturb the placement of the samples. Tape lengthwise along the seam where the foil comes together (we use the same tape that is used to label the plastic bags when the samples are collected). The ends of the packets are left open to facilitate nucleation of free water overnight at -2°C before the programmed freezing run begins. Our freezer accommodates up to 14 of these packets in a single freezer run.

Freezing

Freeze testing requires the use of a freezer where temperatures can be controlled precisely and lowered gradually at a preprogrammed rate. In fact, this is the only expensive (about US\$ 15,000) equipment needed. We use a programmable freezer, Forma Scientific Model 8270/859M with a built-in temperature controller (model West M3750). The packets are placed side-by-side, one layer deep the evening before the run on a thick aluminum shelf in the freezer (to facilitate cooling through conduction rather than convection) and held overnight at -2°C to equilibrate the samples and to freeze extracellular water. Freezing is continued the next morning with temperatures reduced 3°C/hr until the test temperature (see preliminary testing below) is reached (Glerum 1985). After 1 hr at this temperature, the samples are removed from the freezer.

It is important to test the freezer before each use to make sure the controller is working as intended and expected temperatures are reached at the programmed intervals. It is particularly important to test the freezer before the start of the testing season to make sure it is running correctly before samples are collected.

Postfreezing Treatment

Immediately after freezing is completed, the open ends of packets should be sealed by folding (to prevent moisture loss) and the packets refrigerated overnight (for minimum of 12 hr) to allow samples to thaw slowly. The packets are then placed side-by-side (not stacked) at room temperature ($20\text{--}22^{\circ}\text{C}$) for 6–7 days to allow cold injury symptoms to develop (Burr et al. 1990). Sample packets are kept sealed during this period to avoid desiccation and confounding effects that differential sensitivity to light may have on cold injury development.

Damage Scoring

At the end of the fixed storage period at room temperature, each shoot sample is scored for damage. We use an illumi-

nated three diopter magnifying lens for this purpose. It is useful to have freshly cut (i.e., nonfrozen control) samples for comparison. We visually score damage as the percentage of tissue showing injury (yellowing or browning) in 10% classes (i.e., 1 = 0–10%, 2 = 11–20%, . . . 10 = 91–100%). Needle damage is scored as the proportion of total needle surface area (relative to firmly held, fresh green needles) that has browned or fallen off stems. To score stems, a tangential cut is made lengthwise (like peeling a carrot) to reveal approximately 2 cm of cambium and phloem. The proportion of these tissues that has turned from whitish green (undamaged) to yellow or brown is recorded. Buds are also cut lengthwise, and proportion of normally green tissue (including bud needle primordia, bud shoot tissue, and the surrounding bud scales) that is discolored is estimated.

Although visual scoring into damage classes is qualitative, it does not take long before a scorer becomes accustomed to the process. We suggest each scorer start by practicing with a couple of dozen samples, comparing damaged tissues to fresh, undamaged controls, until the eye gets adjusted. If more than one scorer is involved, the scorers should first score a set of samples (20 or so) together to check for consistency. All samples in the same block should be scored by only one individual in order not to confound family differences with scorer bias. Color photos with examples of damage scores for each tissue are available upon request from the senior author.

Preliminary Test

A preliminary test needs to be conducted on a small sample of shoots 1 wk to 10 days before the main test. The purpose of the preliminary test is to establish one or more (usually two) freezing temperatures that will result in intermediate levels of damage in the main test. One shoot for each test temperature should be collected from 15 or so trees scattered throughout the trial being assayed. Preliminary freeze test temperatures (four or more) need to span the range, at about $2\text{--}4^{\circ}\text{C}$ intervals, where intermediate damage might be expected. Table 1 lists preliminary test temperatures that we have used in various cold hardiness assessment experiments, estimated LT_{50} 's for needle, stem, and bud tissues, and the temperatures actually employed in the main tests. Note that at least one temperature utilized in each main test in the fall is slightly lower than the mean LT_{50} 's for the three tissues in the preliminary test. A lower temperature is used to accommodate the ongoing hardening during the week to 10-day lag period between the preliminary and main tests. Similarly, due to ongoing dehardening in the spring, at least one test temperature utilized in each main test in the spring is greater than the mean LT_{50} in the preliminary test. Appropriate temperatures are expected to vary with season, location of test site, and yearly climate. Also, note that temperatures that will inflict intermediate damage in one tissue may be too high or too low for others.

Data Analysis and Sample Size Requirements

Calculating Mean Cold Injury Scores

The goal of freeze testing is to rank families or other genetic entities, such as clones or seed sources, for cold

Table 1. Preliminary test temperatures (°C), estimated LT₅₀s for needle, stem, and bud tissues, and the main test temperatures actually employed in various Douglas-fir cold hardiness studies conducted by the Pacific Northwest Tree Improvement Research Cooperative.

Season/materials	Prelim. test (°C)	LT ₅₀			Main test (°C)
		Needle	Stem	Bud	
Fall (Oct.)					
Seedlings (2 yr old)					
1995	-8, -11, -14, -17	-12.4	-13.5	-15.2	-12, -15
1996	-8, -11, -14, -17	-14	-13	-14	-12.5, -15.5
1997	-8, -10, -12, -14	>-8	-12.7	-9.7	-9, -11
Saplings (4-7 yr old)					
1996	-8, -11, -14, -17	>-8	-12.7	-9.7	-9, -11, -13, -15
1994	-10, -13, -16, -19	-17.5	<-19	<-19	-22.5
Spring (Mar.-Apr.)					
Seedlings					
1997	-10, -14, -18, -22	-19	-18	-20	-15, -19
Saplings					
1997	-12, -15, -18, -21	-21	-18	-16	-12, -14, -16, -18

hardiness. Family differences in cold injury are generally greatest when the mean damage level for a particular test temperature is intermediate (i.e., mean damage score is 3-7 (30-70%)). If two or more freeze temperatures give intermediate damage, their mean score should be used in further analyses because statistical precision of analyses and ability to detect family differences will be increased (Aitken et al. 1996, Aitken and Adams 1996). If, however, a test temperature results in scores that, on average, are either too high (>8) or too low (<2), the data for this temperature should not be included in the analysis.

Data Transformation

Valid statistical tests in analysis of variance may require that the data be transformed prior to analysis (Steel and Torrie 1980). In our experience, if average levels of damage are between 30 and 70%, transformation is usually not necessary. If, however, average damage is high (>70%) or low (<30%), an arcsine square root transformation of injury scores may be required (Sabin and Stafford 1990).

Sample Size Required for Ranking Families and Estimating Heritabilities

A typical coastal Douglas-fir progeny test in the Pacific Northwest is planted with four-tree noncontiguous family plots in each of four or five blocks, although some mortality is expected within the test. This sample size has been sufficient to detect family differences, estimate heritabilities, and rank families for both fall and spring cold hardi-

ness. In many of our coastal Douglas-fir studies, as few as 10 families with 8 trees/family, or about 40 families with 5 trees/family would have been sufficient to detect real cold injury difference among family means with the power of test equal to 0.80 with a 95% confidence interval. As a rule of thumb, 10 to 12 individuals/family should be more than adequate. For additional discussion on sample size estimation, refer to Steel and Torrie (1980).

Cost of Freeze Testing

Outside the cost of a programmable freezer, lab space, minor equipment (e.g., refrigerator, diopter lens, ice chest), and travel costs, the main expense for freeze testing is labor (Table 2). Processing 600 seedlings from a nursery site (e.g., 30 families × 5 replications × 4 trees/replication) at two freeze-test temperatures requires about 72 hr of labor. Collecting cuttings in field tests is more time-consuming (takes up to 115 hr to test the same number of samples) than in nursery tests. Expendable supplies (bags, ice, labels, etc.) for this amount of materials cost around \$100.

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Table 2. Labor required to assess cold injury of 600 nursery seedlings using artificial freeze testing.¹

Activity	Activity details	Person-hours (approx.)
1. Pretest preparation	Data sheet preparation, order expendable supplies, test freezer, plan collection, lineup help, and perform preliminary test.	18
2. Sample collection	Design time schedules for harvesting, testing and scoring samples, organize lab space, collect shoot cuttings.	16
3. Sample preparation	Prepare aluminum foil and cheese cloth, soak cheese cloth, package samples.	2
4. Freezing and postfreeze treatment	Arrange packets inside the freezer, program freezer, conduct freezing, postfreeze packet handling.	6
5. Damage scoring	Score injury damage to needles, stems and buds.	16
6. Data analysis	Data entry, proof reading, analysis. ²	14
	Total person-hours	72

¹ Assumes two test temperatures for each seedling and no travel cost to nursery.

² For ranking families for cold hardiness only (not for preparing reports).

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