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Influence of second flushing on genetic assessment of cold hardiness in coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco)[1]

T.S. Anekonda[a,*], W.T. Adams[a], S.N. Aitken[b]

[a] *Department of Forest Science, Forestry Sciences Laboratory 020, Oregon State University, Corvallis, OR 97331-7501, USA*
[b] *Department of Forest Sciences, University of British Columbia, 193-2357 Main Mall, Vancouver, BC V6T 1Z4, Canada*

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Abstract

The consequences of second flushing for fall cold hardiness in coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco) was investigated in 4-year-old trees from two genetic tests: 42 polycross families in British Columbia and 8 full-sib families in the state of Washington. Cold injury to needle and stem tissues was assessed in October and November in samples of both second-flushed and non-second-flushed shoots from the same trees following artificial freezing. Freeze damage to needles and stems in the second-flushed portion of shoots was 50–60% greater than in non-second-flushed shoots. Great care, therefore, should be taken to consistently sample the same shoot type when comparing cold hardiness of genotypes (or families) using artificial freeze testing. However, because the estimated genetic correlations in freeze injury between second-flushed and non-second-flushed shoots were moderately positive, scoring all trees for hardiness of one shoot type should provide fairly accurate rankings of genotypes for cold hardiness of both shoot types. We recommend scoring non-second-flushed shoots because the frequency of second flushing decreases relatively rapidly with increasing age in coastal Douglas-fir. Hardening of both second-flushed and non-second-flushed shoots was delayed in trees with higher proportions of second-flushed shoots in their crown. Thus, foresters should avoid planting families with high propensity to second flush on high fertility sites (i.e. sites that promote second flushing) susceptible to fall frost events. © 1998 Elsevier Science B.V.

Keywords: Artificial freeze testing; Genetic variation; Heritability

1. Introduction

Shoots of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) trees up to 15 years of age, or so, produce second flushes and continue shoot growth if summer environmental conditions are favorable (Rehfeldt, 1983; Li and Adams, 1993). Genotypes with a propensity for second flushing are generally the fastest in height growth at young ages; however, genotypes that second flush are also more likely to produce forking defects (Rehfeldt, 1983; Adams and Bastien, 1994; Schermann et al., 1998). In addition,

*Corresponding author. Tel.: +1 541 7376579; fax: +1 541 7371393; e-mail: anekondat@fsl.orst.edu

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extended growth in summer may considerably delay the shoot hardening process, rendering second-flushed shoots more vulnerable to damage from late drought or early fall frost (Aitken et al., 1995). The main goal of this study was to evaluate the influence of second flushing on fall cold-hardiness of shoots in coastal Douglas-fir (var. *menziesii*).

Since second flushing leads to the formation of different shoot types with unique developmental stages, it may create problems for assessing cold hardiness in genetic tests. If second-flushed and non-second-flushed shoots have substantially different levels of cold hardiness in early fall (Glerum, 1985), the choice of shoots to sample for artificial freeze testing could greatly influence the results obtained. The presence of second flushing within crowns might also influence fall cold hardiness of other shoots that have not second flushed. Thus, a second goal of this study was to examine the degree to which the proportion of second-flushed shoots on a tree (PSF) influences mean hardiness of both second-flushed and non-second-flushed shoots. Finally, in our previous studies of fall cold hardiness in Douglas-fir, we sampled shoots only from lateral branches for artificial freeze testing (Aitken and Adams, 1995, 1996a, b; Aitken et al., 1995). Susceptibility of the leader shoot to fall cold injury, however, is of great concern because of the negative consequences for stem form and height growth. The final goal of the study, therefore, was to examine the association between leader and lateral shoots in fall cold hardiness.

Specific questions of interest were as follows:

1. To what extent is a second-flushed lateral shoot less cold hardy in the fall than a non-second-flushed lateral? Because the second-flushed shoot has both a portion resulting from the first (primary) flush (SP) and from the secondary flush (SS) (Fig. 1), it was also of interest to determine the degree to which the two portions of second-flushed shoots differ from each other in cold hardiness, as well as from shoots which have not second flushed (NP).
2. To what degree is cold hardiness in different shoot types under similar genetic control?
3. Do trees with a high PSF have lower cold hardiness (over all shoot types) than trees with low PSF?

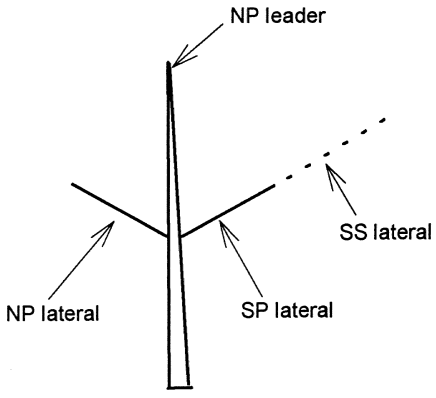

Fig. 1. Diagram of a tree showing different shoot types: Non-second-flushed shoots (NP), and the primary (SP) and secondary (SS) portions of shoots which have second flushed. From each shoot type, needle and stem tissues were scored for cold injury.

4. Does cold hardiness of lateral shoots predict cold hardiness of leader shoots?

2. Materials and methods

Two sets of materials were utilized, each made up of 4-year-old trees planted in farm-field test sites in 1991. The first set consisted of 42 families produced by a polymix mating design (15 males contributed to pollen mix) growing at the Snowdon Seed Orchard on Vancouver Island, British Columbia (BC test). Each family in this test was represented by a four-tree non-contiguous plot in each of eight blocks. Because of high site uniformity, the 12 trees required per family for cold hardiness testing (total N=504) were chosen at random, resulting in one to four trees sampled per family in each of seven blocks.

The second set of materials consisted of eight full-sib families from a test site near Tacoma, Washington (WA test). One to two trees were sampled from each block, so that a total of 6–10 seedlings per family were available from five blocks (N=70). In the BC test no prior information was available on second flushing, but in the WA test, half the families sampled (4) had high average PSF (39.3%; range=16–63%) and half had low average PSF (9.5%; range=0–16%).

Up to three second flushed and three non-second flushed current-year lateral shoots (5 cm long) were collected from each test seedling for cold hardiness

assessment in early to mid-October, 1994. In the WA test, leader shoots in addition to laterals were sampled, and the sampling of both shoots was repeated in early November, 1994 with a different set of individuals from the same eight families. Nearly all leader shoots sampled were the result of a single flush (NP). In both sets of materials, not all trees produced both second-flushed and non-second-flushed laterals. Thus, there was imbalance among families in the number of samples from each lateral shoot-type, as well as imbalance in the number of trees sampled in each block.

Details on the freeze-testing procedures have been presented previously (Aitken and Adams, 1995, 1996a, b; Aitken et al., 1995), so we give only a brief outline here. All shoot cuttings for each test site and sampling period (October and November) were collected on a single day. The cuttings were transported to Corvallis, OR, where on each of three consecutive days, one replicate cutting of each family was put into a programmable freezer (at -2°C) which was slowly reduced in temperature (at 3°C h^{-1}) until one of three predetermined freezing temperatures was reached. After 1 h at the test temperature, the samples were removed, stored overnight at 2°C, and then placed at room temperature for 7 days to allow cold injury symptoms to develop. Preliminary freeze tests were conducted a week before the main tests to determine three test temperatures that would give, on average, intermediate damage levels for each tissue scored. Accordingly, test temperatures used were -10, -13 and -16°C for materials from both sites in October, and -17, -20 and -23°C for the Washington samples in November. Damage to needle and stem (phloem and cambium) tissues of each sample were visually assessed by one individual and scored into 10% classes, based on the proportion of tissue showing injury symptoms (browning).

In the British Columbia samples, needle injury was too high (mean scores >80%) at -16°C and too low at -10°C (mean scores <20%) in all three shoot types, and the family component of variance for injury scores was insignificant at these temperatures. Therefore, only the intermediate needle injury scores (31 to 70%) obtained at -13°C were used in all further analyses of these materials. Stem injury scores, however, were intermediate and their family components of variance were significant at both -16 and -13°C for the British Columbia samples, hence, the average of scores obtained at these two temperatures were used in the analysis of stem tissue. Both needle and stem injury scores were averaged over all three temperatures in the Washington cuttings, as injury scores were intermediate and family components of variance were significant at all test temperatures.

3. Statistical analyses

3.1. British Columbia samples

Paired *t*-tests (MEANS procedure, SAS Institute Inc., 1989) were used to test mean differences in cold injury scores between shoot types (i.e. NP, SP, and SS) for needles and stem tissues separately (Question 1). The minimum number of pairs involved in any one comparison was 222. All tests of significance in this study were done at the 0.05 probability level.

Analyses of variance were conducted on injury scores of each tissue assuming the following linear, random model:

$$Y_{ijk} = \mu + b_i + f_j + bf_{ij} + e_{ijk}$$

where, Y_{ijk} is the individual-tree value for each trait, μ the experimental mean, b_i the random effect of the ith block, f_j the random effect of the jth family, bf_{ij} the random interaction effect of the jth family in the ith block, and e_{ijk} the random tree error of kth tree in the jth family of the ith block. Analyses first employed the GLM procedure of the SAS statistical software package (SAS Institute Inc., 1989) in order to test the significance of family differences (Type III sums of squares). Components of variance in the model were then estimated using the restricted maximum likelihood (REML) method of the SAS VARCOMP procedure. Additive genetic variance was calculated as four times the family variance (Squillace, 1974; Falconer, 1989). Individual tree heritabilities (h^2) (Question 2) and their approximate standard errors were estimated following Namkoong (1981), and Dickerson (1969), respectively.

Genetic correlations (r_A) between injury scores in different shoot types (Question 2) were estimated following Burdon (1977):

$$r_A = \frac{\mathrm{Cov}_{AB}}{\sqrt{(\sigma_A^2 \times \sigma_B^2)}}$$

where Cov_{AB} is the estimated family covariance of injury scores between shoot type A and shoot type B, and σ^2_A and σ^2_B the estimated family variances of the injury scores of the two shoot types. Cov_{AB} was derived by conducting an analysis of variance of A+B (REML method), and employing the well-known relationship: $Cov_{AB} = (\sigma^2_{(A+B)} - \sigma^2_A - \sigma^2_B)/2$.

3.2. Washington samples

Differences between high and low PSF families in injury scores for each shoot type (Question 3) were tested for each sampling date using Cochran's approximate '*t*'-statistic for unequal variances (TTEST procedure, SAS Institute Inc., 1989). In addition, simple individual phenotypic correlations between the leader and non-second-flushed laterals were estimated (Question 4). In order not to confound these correlations with propensity level (i.e. high vs. low PSF), the correlations were calculated within each propensity level and then averaged. Individual scores were then adjusted for their corresponding propensity-level means, and the residual scores of leaders and laterals were plotted to visualize the degree of individual-tree association of these shoot types.

4. Results and discussion

4.1. Needle and stem injury in different shoot types

Cold injury results from the British Columbia test clearly demonstrate that second-flushed shoots are less cold hardy in early fall than non-second-flushed shoots (Fig. 2). There is an intriguing difference, however, between needle and stem tissues in the degree to which hardiness of the primary portion of second-flushed shoots (SP) is delayed by second flushing. Hardiness of SP needles seems to be little affected by second flushing, because cold injury of these needles is nearly the same as needles on non-second-flushed shoots (NP). Cold injury of SP shoots, however, is nearly the same as the second-flushed portion (SS) of the same shoot, indicating that second flushing delays shoot hardening of both the primary and secondary flushes. This difference in pattern of hardiness between needles and stems may be attributed to cambial activity and physiological changes that occur

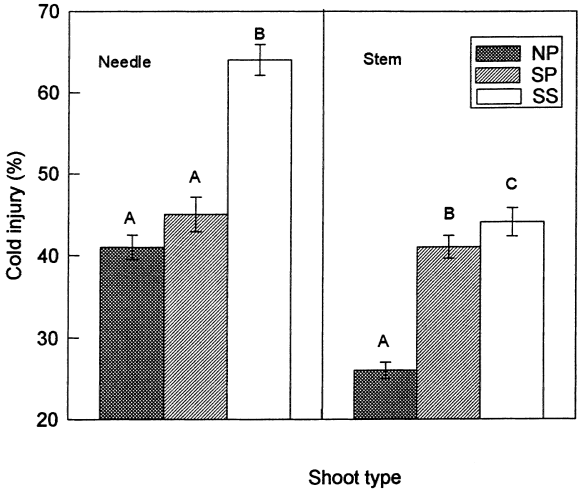

Fig. 2. Estimated mean cold injury to needle and stem tissues in three shoot types (NP, SP, SS; see Fig. 1) of laterals sampled from British Columbia families. Error bar is the standard error of the mean. When letters over bars differ, mean cold injury scores between shoot types differ significantly ($p<0.05$).

during second flushing and tree hardening (Sakai and Larcher, 1987; Dickson, 1991). We offer two alternative, although not mutually exclusive hypotheses, which are speculative, but may be of value in designing future studies. First, hormones produced in expanding buds of SS are transported basipetally to the SP stimulating increased cambial activity. It is likely that the primary growth-stimulating hormone is indole-3-acetic acid (IAA), which has been isolated from developing shoots and xylem sap of Douglas-fir (DeYoe and Zaerr, 1976), although giberellins also appear to play a role in cambial division and differentiation (Little and Pharis, 1995). Active cambial cells and newly produced phloem do not develop cold hardiness as quickly as tissues that cease growing earlier (Sakai and Larcher, 1987). Thus, stems of both SS and SP are expected to be less hardy in early fall, than stem tissues on NP, which are physically further away from the sites of late-season hormone production. Needles in SP are not as influenced by growth promoting hormones from SS because needles are nearly or completely fully developed at the time of second flushing, and also they do not possess cambial meristem.

The second hypothesis is based on the idea that accumulated carbohydrates (e.g. reducing sugars) and

other compounds (e.g. proteins) may function as cryoprotectants in dormant plant tissue and facilitate tissue hardening in fall and winter months (Kruger and Trappe, 1967; Kramer and Kozlowski, 1979; Ho, 1988; Alberdi and Corcuera, 1990; Omi, 1990; Dickson, 1991). Translocation of these compounds from SP to facilitate growth in SS lowers their concentration in SP, slowing the development of stem hardening. These compounds might also be translocated to SS from NP stems, but probably to a lesser degree. Thus, carbohydrate concentration (and fall hardening) is expected to be higher in NP stems, than in stems of either SS or SP. Cold hardiness of mature needles in SP may be little influenced by second flushing on the same stem because they are further removed from SS in the sap stream, and thus contribute less to carbohydrate accumulation and mobilization.

4.2. Genetic control of cold hardiness in different shoot types

Family ranges in mean cold injury scores were large for both needles and stems in the British Columbia test, and family variances were significant ($p<0.05$) in all cases except needle damage on non-flushed shoots (Table 1). Estimated individual heritabilities for stem cold injury were low for all shoot types, consistent with the low heritabilities for fall cold injury of stems revealed in our earlier studies of coastal Douglas-fir breeding populations in Oregon and Washington ($h^2=0.30$; Aitken and Adams, 1996a; Aitken et al.,

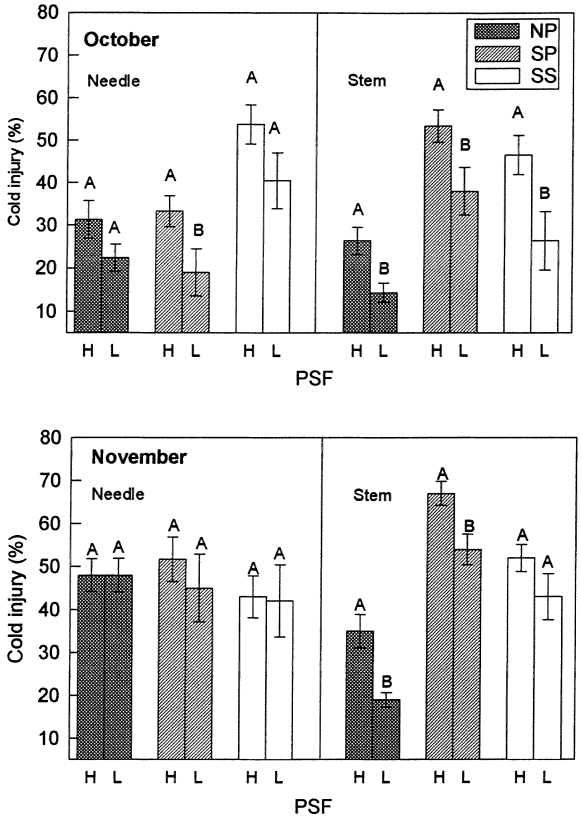

Fig. 3. Influence of high (H) vs. low (L) proportion of second-flushed shoots in the crown (PSF) on mean fall cold injury to needle and stem tissues from three shoot types (NP, SP, SS; see Fig. 1). Error bar is the standard error of the mean. When letters over paired H and L bars differ, mean cold injury scores differ significantly ($p<0.05$).

Table 1
Estimated individual heritabilities (h^2) for cold injury in needle and stem tissues of three shoot types, ranges over family means [a], and genetic correlations between traits for trees in a British Columbia farm-field test.

Trait	Shoot type [b]	h^2 [c]	Family range	Genetic correlations	
				SP	SS
Needle injury	NP	–[c]	16–65	–[c]	–[c]
	SP	0.75	8–95	–	0.70
	SS	0.75	23–95	0.70	–
Stem injury	NP	0.15	13–73	–[d]	0.34
	SP	0.31	9–69	–	0.45
	SS	0.18	5–87	0.45	–

[a] Forty-two families.
[b] See Fig. 1 for description of shoot types.
[c] Could not be estimated because family differences were not significant ($p>0.05$).
[d] Estimate >1.

1995). Heritability estimates for needle injury were greater than we observed previously (h^2<0.40), but could only be calculated for second-flushed shoots. In our earlier assessments of cold injury we sampled primarily NP shoots (ca. 95% of the time), because the trees were at least 7 years old and the frequency of second flushing was never great.

It is clear from Fig. 2 that all families must be sampled for the same shoot type (or the same mix of shoot types) if valid comparisons of cold hardiness are to be made among families. If all families are sampled for one shoot type, however, to what extent can cold injury to other shoot types be predicted? This is largely a function of the magnitude of genetic correlations between cold injury scores for the different shoot types, which ranged from low to relatively strong for different comparisons (Table 1), and averaged 0.50. It appears from these results that family rankings for fall cold hardiness based on one shoot type should at least be roughly comparable to those based on samples from another shoot type.

4.3. *Influence of proportion of second-flushed shoots in the crown (PSF) on cold hardiness of individual shoots*

The influence of PSF on cold injury of individual shoots was evaluated in the Washington samples (Fig. 3). It is clear that susceptibility of shoots of all types to cold injury is greater in families with high

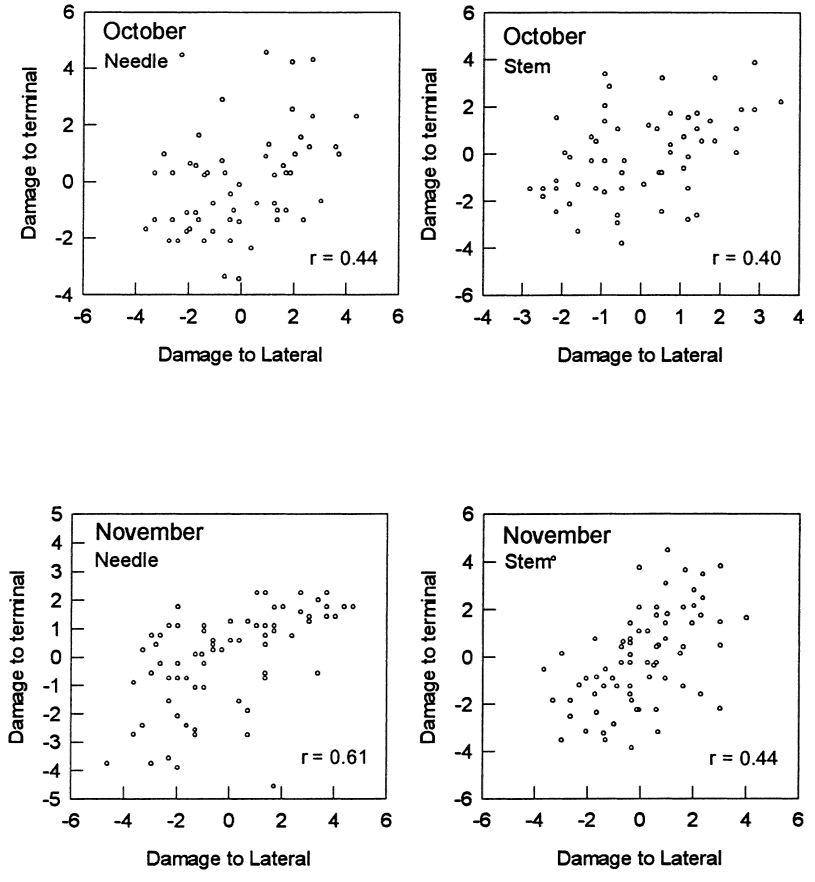

Fig. 4. Scatter plots of individual cold injury scores (in October and November) for needle and stem tissues from leader vs. lateral shoots of the same tree. Scores are for non-second-flushed laterals, since leaders did not second flush. Individual-tree scores were adjusted prior to plotting for cold injury level associated with high vs. low proportion of second-flushed shoots in the crown (PSF) (see text).

PSF than low PSF in October, but differences in susceptibility decrease, especially in needle tissue, by November. Thus, at least in early fall, families producing a high proportion of second-flushed laterals are particularly susceptible to frost damage; not only because second-flushed shoots are less hardy themselves, but because the higher frequency of second-flushing delays hardening of non-second-flushed shoots, as well. It should also be noted that needles and stems followed patterns of cold injury across shoot types similar to those observed in the BC samples (compare Figs. 2 and 3). The influence of PSF on overall cold hardiness seems consistent with both the hormone cambial-growth initiation and carbohydrate mobilization hypotheses mentioned earlier. Higher PSF means both needle and stems of all tissue types are likely to experience later growth cessation and/or slower carbohydrate storage build-up making all shoots more susceptible to cold damage in early fall.

4.4. Predicting cold hardiness of leader shoots from injury scores on laterals

The Washington samples were also utilized in evaluating the ability to predict cold hardiness of leader shoots from cold hardiness of laterals. Because leaders in this test did not second flush, we compared cold injury in leaders with cold injury in non-second-flushed laterals. Individual-tree correlations between leader and lateral cold injury scores were consistent and positive across tissue types and months, but not large in magnitude (mean r=0.47) (Fig. 4). The results suggest that ranking individuals for leader cold hardiness on the basis of cold hardiness in laterals should be fairly reliable.

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