Comparison of Four Cold Hardiness Tests on Three Western Conifers[1]

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Abstract.--Container-grown ponderosa pine, Douglas-fir, and Engelmann spruce seedlings were cold acclimated and deacclimated in growth chambers over 19 weeks. A wholeplant freeze test and three tissue tests were performed weekly. The whole-plant freeze test provided results in 7 days and indicated differences in cold hardiness between stems, buds, and needles. Results from a freeze-induced electrolyte leakage test and differential thermal analysis were available in 2 days and 1 hour, respectively. Both tests were good predictors of tissue cold hardiness when calibrated against the whole-plant freeze test. Ethylene and ethane evolution were poor predictors of the development of cold hardiness.

INTRODUCTION

The ability to measure cold hardiness is necessary for successful production and establishment of high-quality greenhouse and field-grown tree seedlings (Glerum 1985, 'Warrington and Rook 1980). It provides an essential tool for developing optimum greenhouse hardening regimes and for determining the timing of removal of stock from greenhouse to storage or field environments. It provides critical information for sound decisions regarding the timing of lifting, storage and handling, and outplanting of bareroot stock. Knowledge of cold hardiness can also substantially reduce losses from late spring and early fall frosts by establishing the need for protective measures.

The primary method used to assess seedling cold hardiness is the whole-plant freeze test (Ritchie 1984). Though the test is highly reliable, it is time-consuming and the results are not available for at least 7 to 14 days, which is not as soon as usually needed. There are, however, physiological parameters which can be conveniently measured and utilized as tissue tests for cold hardiness that can provide results within 1 to 2 days, or as soon as 1 hour, depending on the test. If a consistent relationship exists between the results of the whole-plant freeze test and one or more tissue tests, seedling cold hardiness could be measured quickly. The reliability of the results from a tissue test would then depend on the calibration, or adjustment, of the tissue test results to match the whole-plant freeze test results.

This paper describes our evaluation of three tissue tests: freeze-induced electrolyte leakage, differential thermal analysis, and ethylene and ethane evolution. Each is quick, objective, and non-destructive of whole plants. Each has its strengths and weaknesses, however, and none are universally applicable. The purpose of this study was to assess the usefulness of these three tissue tests for predicting cold hardiness, and to make the first calibrations of the tissue tests against the whole-plant freeze test.

MATERIALS AND METHODS

Ponderosa pine (*Pinus ponderosa* Laws.), interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco), and Engelmann spruce (*Picea engelmannii* (Parry) Engelm.) were greenhouse container grown, then cold acclimated and deacclimated in growth chambers over a 19-week regime as described by Tinus et al. (1986). At weekly intervals, samples of seedlings were taken for whole-plant freeze tests and root growth capacity

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[1]Paper presented at the Western Forest Nursery Council Meeting. [Tumwater, Wash., August 12 - 15, 1386].

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and bud dormancy tests. To establish a firm relationship between the whole-plant tests and the three tissue tests, all were done concurrently each week. An upper lateral branch or a pair of fascicles, depending upon the species, was removed from each of 20 trees per species in the weekly sample for use in the tissue tests, and then the seedlings were used for the whole-plant tests.

Whole-Plant Freeze Test

The procedures for the whole-plant freeze test and subsequent analysis were as described by Tinus et al. (1986). In addition to measuring percent injury to stem tissue in the whole-plant freeze test, percent injury to buds by a count of live and dead buds, and percent injury to needles by visual estimation of browning, were also measured after 7 days to provide a reference for comparison of the three tissues. The 50% injury points for the three tissues were estimated by regression and calibration methods, following pooling of the data by rate of injury across species, days, and tissue types.

Freeze-Induced Electrolyte Leakage

Measurement of electrolyte leakage from stressed tissue to assess viability is a technique developed by Dexter et al. (1930, 1932). When plant tissue is cooled to temperatures that cause injury, cell membranes are disrupted and electrolytes leak out. The greater the injury, the greater the leakage.

Needle segments, 1 cm long, cut at both ends, were prepared from the tissue samples pooled from the 20 trees per species each week. Segments were washed in distilled water and transferred in random groups of 10 to culture tubes containing 0.5 ml distilled water. Three control tubes per species were stoppered and placed in a refrigerated icewater bath at 1°C. Treatment tubes, 21 pe species, were placed in a Forma Scientific[3] methanol bath at -2°C. After 0.5 hour the water in the treatment tubes was nucleated with a -80°C wire and the tubes were stoppered. The methanol bath was then cooled at the rate of 5°C per hour. At each of seven test temperatures, selected to span 0 to 100% injury, three treatment tubes per species were removed to thaw in the ice-water bath. After all the tubes were removed from the methanol bath and thawed, 3 mls of distilled water were added to each of the 24 tubes per species and all tubes were stoppered and placed in a 100 rpm shaker at 24°C for 20 hours incubation. Conductivity of the solution in each tube was measured after incubation, and the tubes were then placed in a boiling water bath for 10 minutes to induce complete tissue injury. Conductivity was remeasured after an additional 20 hours incubation in the shaker.

[3]Trade names are used for brevity and specificity and do not imply endorsement by USDA or Colorado State University to the exclusion of other equally suitable products.

Test results, which were available in 2 days, were reported as percent index of injury (I), calculated by the formula

$$I = 1 - \frac{1-(T_1/T_2)}{1-(C_1/C_2)} \times 100$$

where T_1 and T are the conductivity of the treatment solution before and after boiling, respectively, and C, and C are the conductivity of the control solution before and after boiling, respectively (Flint et al. 1967). The formula adjusts the leakage resulting from the low temperature stress for the leakage from unstressed controls and for the total leakage possible with complete injury of the individual samples of needle segments. The greater the index of injury, the less the cold hardiness at a test temperature.

A weekly data set consisted of 21 observations (three replicates at seven temperatures) for each species. A modified Gauss sigmoid model (Grosenbaugh 1965) was fitted to each set of 21 points (except Engelmann spruce data from days 84, 98, and 105 where linear regression was used) such that

$$I = b_1(1-b_2e^{-b_3t^{b_4}}),$$

where t represents temperature. Temperatures at various index of injury levels were estimated by inverting these models.

Differential Thermal Analysis

The cold hardiness of some tree species is related to a capacity for supercooling, or the cooling of water below the freezing point without ice formation (Burke et al. 1976). The extent of supercooling can be measured by differential thermal analysis. The profile of a cold-hardened bud that supercools (Burr et al. 1986) has two peaks or exotherms representing heat released by the freezing of water within the bud. The first exotherm represents freezing of extracellular water at approximately -6°C, which generally causes no injury to the bud. The second, or lowtemperature, exotherm represents freezing of deep supercooled intracellular water and is associated with lethal injury (Sakai 1978). The temperatures at which low-temperature exotherms occur have been correlated with bud acclimation and deacclimation to cold (Tinus et al. 1985). This test can be used with members of many gymnosperm and angiosperm genera such as Abies, Acer, Carya, Fraxinus, Gleditsia, Juniperus, Larix, Picea, Pseudotsuga, Quercus, Tsuga, and Ulmus (Becwar 1980, George et al. 1974, Sakai 1978, 1979). In non-supercooling genera such as Pinus, however, the differential thermal analysis profile is of no diagnostic value because there is no low-temperature exotherm.

Nine well-developed buds of both Douglas-fir and Engelmann spruce were randomly selected each week from the pooled tissue samples for differential thermal analysis. Buds were excised from the stem tissue below the base or crown of the bud to ensure the integrity of morphological features promoting

supercooling (Sakai 1979). The cut surface of an excised bud was wetted with distilled water and dipped in a powdered synthetic mica nucleator to reduce supercooling of extracellular water. The bud was then placed in a small cylinder of aluminum foil. The foil cylinder was crimped around a copper-constantan differential thermocouple to insure contact between the thermocouple and bud. A similar foil cylinder without a bud was placed around a reference thermocouple. Several differential thermocouples and the reference were individually covered with small plastic centrifuge tubes and then placed in an aluminum block, which provided uniform cooling. The aluminum block, wrapped with heat tape to regulate temperature, was cooled in a -80°C freezer at 1°C per minute. The cooling rate was automatically controlled by a microprocessor-based controller connected to a variable transformer. Cooling of the block and freezing of water within the buds were documented by wiring the thermocouples through amplifiers and into strip chart recorders.

The aluminum block could be fitted with buds, assembled, cooled, and the data extracted from the strip charts within 1 hour. Mean low-temperature exotherm temperatures with 95% confidence intervals were calculated from each group of nine buds per species each week.

Ethylene and Ethane Evolution

Intact plant tissues synthesize ethylene at different rates during the various developmental stages of the annual growth cycle, with rates highest during active growth periods and lowest during dormancy (Seibel and Fuchigami 1978). Ethylene evolution from excised tissue harvested on different dates has been related in the same manner to the level of vegetative maturity and hardiness in white pine. Ethylene levels were highest in spring during active growth, declined to low levels in fall with vegetative maturity, and were not detectable during winter. Ethylene and ethane evolution have also been found to rise under conditions of stress and strain, respectively (Harber and Fuchigami 1986). The evolution of these two gases was examined not as a viability test, but to explore the hypothesis that the seasonal pattern of evolution of one or both might provide a predictive tool for determining the beginning and ending points of the winter dormancy period, or the starting of acclimation and deacclimation to cold.

Ponderosa pine needle tissue was used for the ethylene and ethane evolution test. Needle tip segments, 2 cm long, were prepared from the tissue samples pooled from the 20 trees each week. Segments were randomly placed into 10, 2 cc gastight serum vials, 10 segments per vial. Vials were incubated for 24 hours at 24°C in darkness. Following incubation, a 1 cc gas sample was taken from each vial using a gas-tight syringe, and analyzed for both ethylene and ethane using an Perkin-Elmer 990 gas chromatograph. An activated alumina column was used, and injection port, column, and detector temperatures were 75 C, 50 C, and 150°C, respectively. Preliminary results were available shortly after injections were completed, and final results, reported as ppm/mg dry wt./hour, were available after a 48 hour oven drying period.

Box plots were used to flag outliers in the final data set (Chambers et al. 1983). Six observations that were several, often more than 10, standard deviations from the mean were omitted after each was found to be biologically unreasonable or indicative of problems in experimental procedures Mean rates of ethylene and ethane evolution with 95% confidence intervals were calculated for each week from the remaining observations.

RESULTS AND DISCUSSION

Whole-Plant Freeze Test

During acclimation and deacclimation, changes in the cold hardiness of needle and bud tissue of the three species followed the same general patterns as stem tissue (fig. 1). Ranking of the tissue types by maximum cold hardiness attained was the same in all three species when tissue differences occurred. Stem tissue achieved the greatest cold hardiness. Needle tissue cold hardiness was similar to that of stems during the first two stages of acclimation and during deacclimation, but did not reach the very hardy **levels** that stems did. Buds were consistently the least cold hardy of the three tissues in all three species.

The rate of cold hardening in all three tissues of ponderosa pine (fig. 1A) appeared to level out in the third stage of the regime, while in Douglas-fir (fig. 1B), only the hardening of bud and needle tissue stabilized. In Engelmann spruce (fig. 1C), there was no indication that maximum cold hardiness was reached in any of the three tissues.

Needle tissue may be slightly more cold hardy than stem tissue in Douglas-fir and Engelmann spruce during the last 2 weeks of deacclimation, but not in ponderosa pine. The last flush of growth in Douglas-fir was readily distinguishable from earlier growth, before the onset of hardening. During this time, the newest needles were significantly less cold hardy than older needle tissue, at the 95% level of confidence.

The importance of assessing the cold hardiness of the tissue types separately because of differential hardening, such as described here, has been recognized by other researchers (Blake et al. 1979, Timmis 1977). The differential hardening of tissues has a critical impact on the assessment of economic viability of planting stock. For this reason the Industrial Forestry Association has also incorporated separate ratings of needle, bud, and stem injury following freezing tests into their operational cold hardiness testing program (Glerum 1985).

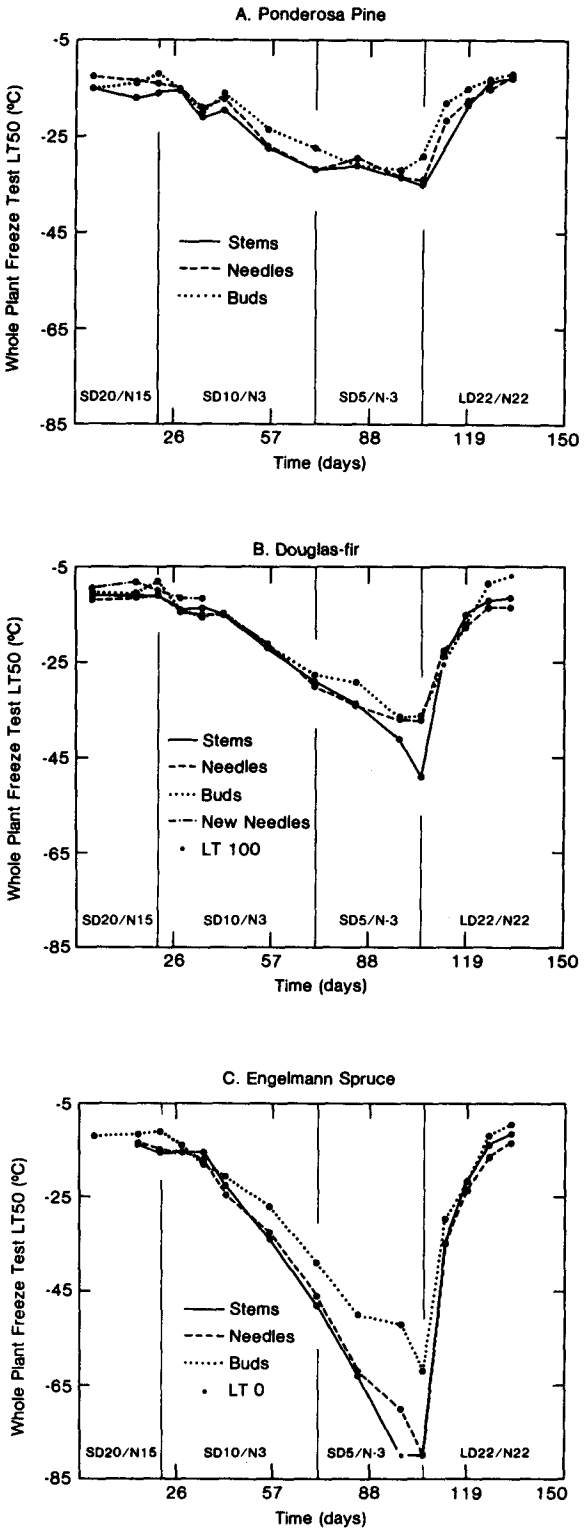


Figure 1.--Stem, bud, and needle cold hardiness of (A) ponderosa pine, (B) Douglas-fir, and (C) Engelmann spruce as a function of time, determined by the whole-plant freeze test. Growth chamber conditions are indicated across the bottom of each graph and are described in Table 1 of Tinus et al. (1986).

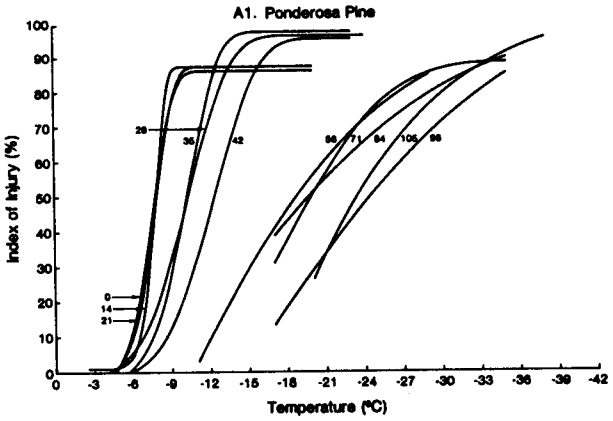
Freeze-Induced Electrolyte Leakage

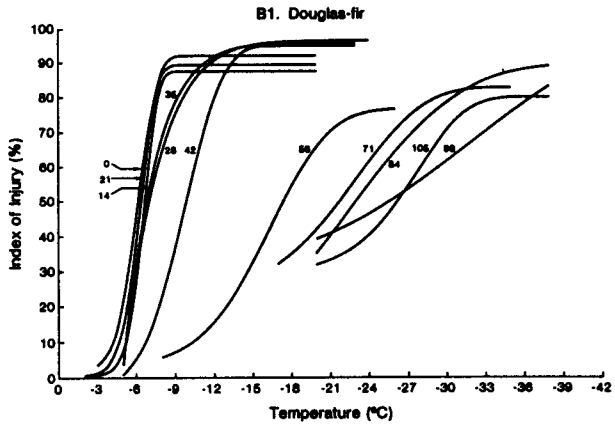
The weekly models of percent index of injury of needle tissue as a function of stress temperature for the three species (fig. 2) are labeled by the day number on which the test was run. Models from days 0, 14, and 21 were from the first stage of acclimation; models from days 28, 35, 42, 56, and 71 were from the second stage; the third stage included models from days 84, 98, and 105; and the remaining models, 112, 119, 126, and 133, were from the deacclimation stage. The range in R^2 for all modified Gauss sigmoid models was .998 to .874. The three linear models, Engelmann spruce days 84, 98, and 105, had R values of .867, .693, and .669, respectively.

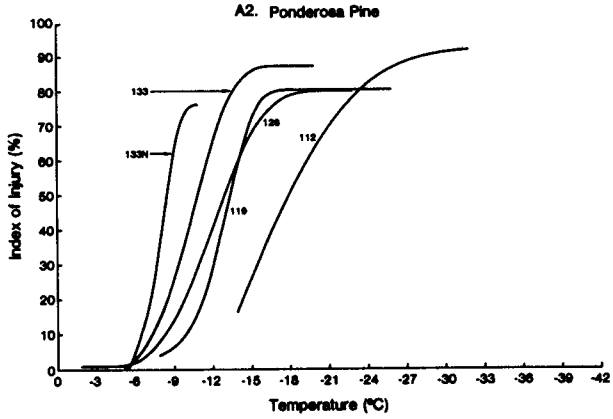
Statistical differentiation of the models has not been completed, but preliminary indications are that the test can detect significant differences in cold hardiness of just a few degrees C, at the 95% level of confidence. After little change during the first stage of the regime, there was a gradual progression in cold hardening to day 105, the last data set before deacclimation. The proportionately large loss of cold hardiness during the first week of deacclimation was indicated by a similar change in index of injury from day 105 to 112. Deacclimation then proceeded at a slower rate to day 133 at the end of the 19 weeks. The difference in cold hardiness between new growth and the previous season's growth, after bud break at the end of the 19 weeks, was readily detectable. The new growth, model 133N, was less hardy than the previous season's growth, model 133, in all three species. Once hardened, the previous season's needles did not deharden by day 133 to the level of the new tissue, but retained some residual cold hardiness.

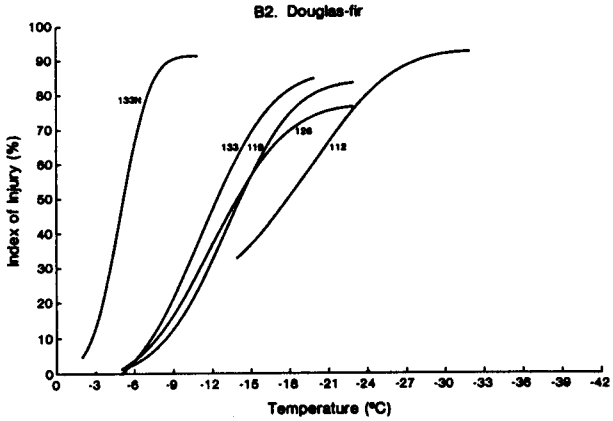
There was also a gradual change in the shape of the models with acclimation and deacclimation. For minimally hardy seedlings (e.g., days 0, 14, 21, and 133), any decline in temperature produced a large increase in injury. As plants became more hardy (e.g., days 84, 98, and 105), a similar decline in temperature produced less injury. This change in sensitivity to declining temperature is an important consideration when assigning critical minimum temperatures to seedlings.

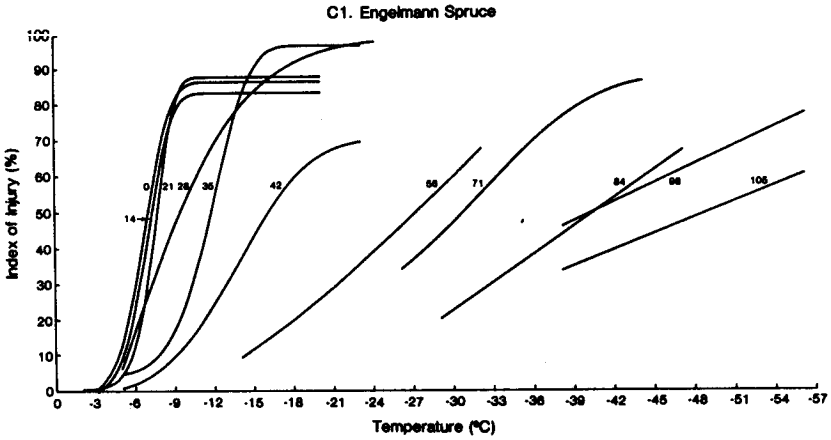
The stress temperatures resulting in 50% index of injury of needles each week were estimated from the data in figure 2 and compared to the whole-plant freeze test temperatures resulting in 50% needle injury (LT_{50}) to examine the relationship between the results of the two tests (fig. 3). The freeze-induced electrolyte leakage test followed the changes in cold hardiness as indicated by the whole-plant freeze test quite well for all three species. However, with the exception of the last week of deacclimation, the freeze-induced electrolyte leakage test was a more conservative test because 50% index of injury occurred at a higher temperature than did 50% injury in the whole-plant freeze test. Calibration of the freeze-induced electrolyte leakage test was necessary because of this difference. Preliminary calibration indicated that the











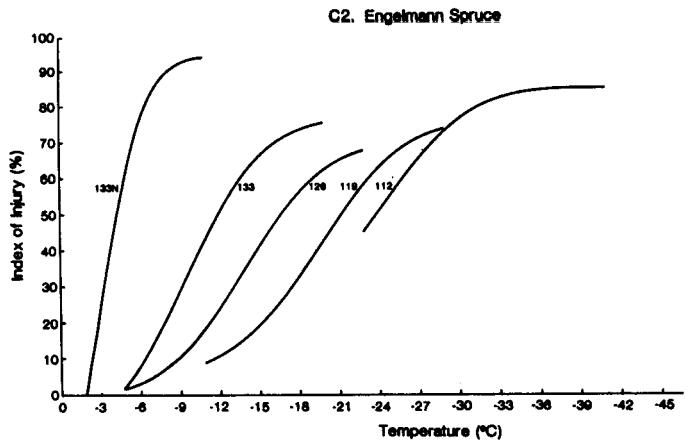


Figure 2.-- Weekly models of index of injury as a function of stress temperature for (A) ponderosa pine, (B) Douglas-fir, and (C) Engelmann spruce determined by the freeze-induced electrolyte leakage test on needle tissue. Graphs Al, B1, and Cl show the weeks of acclimation, and graphs A2, B2, and C2, the weeks of deacclimation. Models are labeled with the day number on which the test was run, beginning with day 0 and ending with day 133. Model 133 N represents new needle tissue produced following bud break.

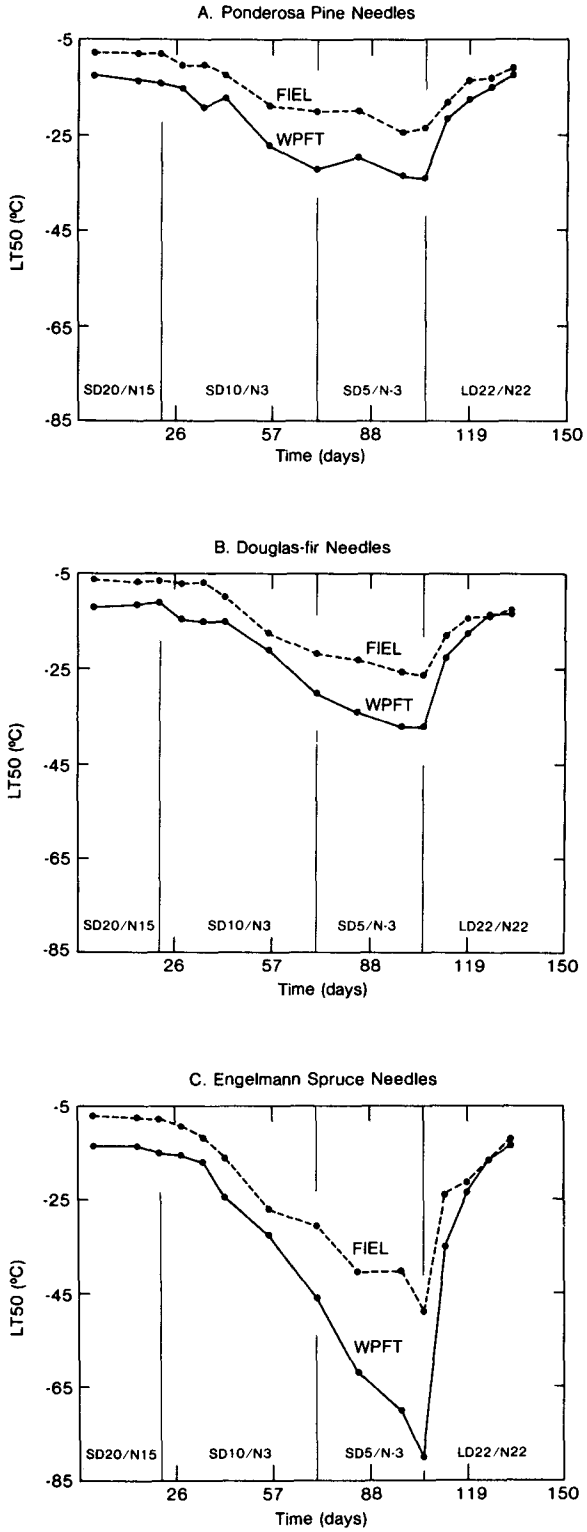


Figure 3.--Temperatures resulting in 50% index of injury to needle tissue in the freeze-induced electrolyte leakage test (FIEL) and 50% needle browning in the whole-plant freeze test (WPFT) as a function of time for (A) ponderosa pine, (B) Douglas-fir, and (C) Engelmann spruce. Growth chamber conditions are indicated across the bottom of each graph and are described in Table 1 of Tinus et al. (1986).

stress temperature resulting in 70 to 90% index of injury in the freeze-induced electrolyte leakage test frequently matched the whole-plant freeze test LT_{50} during acclimation.

This test has been used by a number of workers on a range of coniferous tree species, including Scots pine (Pinus sylvestris)(Aronsson and Eliasson 1970), Monterey pine (Pinus radiate) (Green and Warrington 1978), Douglas-fir (Psuedotsuga menziesii)(van den Driessche 1969, 1976), and black spruce (Picea mariana) and white spruce (Picea glauca)(Colombo et al. 1982). These workers have reported a close relationship between electrolyte leakage from tissue samples and longer term development of visible injury symptoms of intact plants exposed to whole-plant freeze tests. Attempts to predict the lethal temperature of whole plants subjected to freezing tests based on electrolyte leakage of composite tissue samples (e.g. entire stem sections with needles and buds attached) met with variable success. Calibration of electrolyte leakage from a particular tissue to the response of that same tissue in a whole-plant freeze test has not been previously reported.

Differential Thermal Analysis (DTA)

The average weekly low-temperature exotherm temperatures of Douglas-fir and Engelmann spruce buds were compared to the whole-plant freeze test bud LT 's to examine the relationship between the results of these two tests. The DTA test results for both species followed the changes in cold hardiness as indicated by the whole-plant freeze test, but DTA was a consistently more conservative test (fig. 4). DTA data were not available during early acclimation, when bud cold hardiness was warmer than approximately -6°C, because any lowtemperature exotherm was masked by the first exotherm.

Based on a sample size of nine, the precision of the low-temperature exotherm means allowed differences during acclimation of t 2°C to be statistically detected (p=.95). Differences of ± 3 to 4°C were significant during deacclimation when the change in cold hardiness was more rapid.

In preliminary calibrations of DTA to the whole-plant freeze test, the average temperature at which the low-temperature exotherm occurred corresponded well with the lowest temperature at which no visible injury to buds occurred, or an LT , in the whole-plant freeze test (Tinus et al. 1996). Since the buds were the least hardy of the tissues, the average low-temperature exotherm temperature represented the lowest temperature at which there was no injury to the seedling as a whole.

Ethylene and Ethane Evolution

There was a general decline in the evolution of both ethylene and ethane from ponderosa pine needle segments during the 19-week regime (fig. 5). However, the data were so variable that both were poor predictors of seedling or tissue cold

hardiness. Some interesting patterns were evident, though. Each time the environmental conditions changed, ethylene evolution was stimulated. The peak in the first stage of acclimation followed the move of the trees from the greenhouse to the growth chambers, and the remaining three peaks occurred at the start of each of the subsequent stages. Greenhouse temperatures averaged approximately 26°C during the day and 20°C at night, with day length extended to 22 hours with flores-cent light. The change from greenhouse to growth chamber conditions was comparable in magnitude to

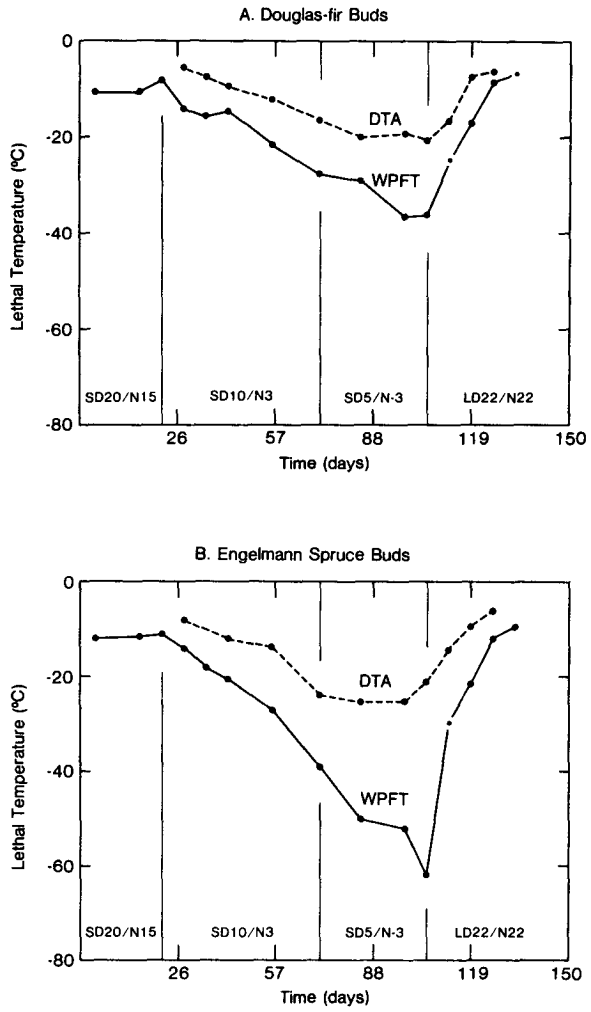


Figure 4.--The average low temperature exotherm temperatures from differential thermal analysis (DTA) of buds and the temperature resulting in 50% bud mortality in the whole plant freeze test (WPFT) as a function of time for (A) Douglas-fir, and (B) Engelmann spruce. Whole-plant freeze test data for Douglas-fir on day 112 represent an LT_{100} at -25 C, and on day 133 represent an LT at -7°C. Whole-plant freeze test data for Engelmann spruce on day 112 represent an LT10Y at -30°C. Growth chamber conditions are indicated across the bottom of each graph and are described in Table 1 of Tinus et al. (1986).

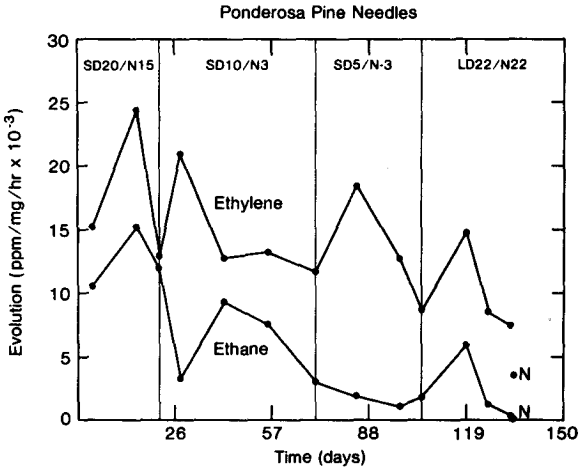


Figure 5--Ethylene and ethane evolution from ponderosa pine needle segments as a function of time. Data points labeled 'N' represent evolution from new growth produced following bud break. The upper point is ethylene, the lower, ethane. Growth chamber conditions are indicated across the top of the graph and are described in Table 1 of Tinus et al. (1986).

the other changes. Though a rise in ethylene evolution may be an indicator of environmental change in general for ponderosa pine, the large standard errors of the means made detecting statistically significant differences over time difficult with the sample size of 10 that was used. Differences in ethylene evolution from new growth produced following bud break and the previous season's growth were not statistically significant (fig. 5).

The ethane evolution means were much more precise, and all the departures in the data from the general declining trend were significant at the 95% level of confidence. There was a large drop in ethane evolution as the trees began to cold harden in the second stage, and a large rise in ethane evolution as the trees began deacclimation in the fourth stage. Thus, ethane evolution may be a good indicator of the start of acclimation and deacclimation in ponderosa pine, even though the trees show no visible signs of these turning points in development.

CONCLUSION

The four cold hardiness tests are compared in Table 1. The whole-plant freeze test was most accurate, and could conceivably be used with all species, but the test was cumbersome, destructive of whole plants, and time consuming. Operationally, reliable whole-plant freeze test estimates would also require more plants than used here because of increased viability in field-grown crops.

With the species we tested, the freeze-induced electrolyte leakage test and differential thermal analysis were good, objective predictors of tissue

cold hardiness when calibrated to the whole-plant freeze test. They were both precise and thus detected slight changes in cold hardiness. The freeze-induced electrolyte leakage test, though it required two, 20-hour incubation periods, could be tested for use with all conifers, and a great many samples could be measured concurrently with no increase in equipment. It is already being used operationally, with modified methods, because of these advantages (Colombo et al. 1984). The main advantage of differential thermal analysis is that the results can be available within an hour. Buds are also very convenient to sample, require minimal preparation, and exhibit well-defined exotherms. However, the test can only be used with species that deep supercool, and only when reasonably well-developed buds are present. Additionally, differential thermal analysis requires complex equipment which is not readily expandable to handle large sample sizes.

Ethylene and ethane evolution from needle tissue were both poor predictors of cold hardiness in ponderosa pine, but further research may show ethane evolution to be a good indicator of the start of acclimation and deacclimation in this species. The usefulness of this test for assessing the development of cold hardiness in other species has yet to be investigated. The major advantage of such a test is that the tissue sampled requires no exposure to stress temperature treatment.

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Table 1.--Comparison of four cold hardiness tests.

	Predictive ability	Species applicability	Tissue needed	Time required
Whole plant freeze test	excellent	all	whole plant	7-14 days
Freeze induced electrolyte leakage	good	all conifers	needles	2 days
Differential thermal analysis	good	only supercooling	buds	1 hour
Ethylene and ethane evolution	poor	unknown	needles	1 day

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