COLD ACCLIMATION AND FREEZING IN DOUGLAS-FIR SEEDLINGS

by

ROGER TIMMIS
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Department of Forestry

The University of British Columbia
Vancouver 8, Canada

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ABSTRACT

Five major investigations were conducted on the cold hardiness of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) seedlings: (1) to define a hardiness measurement technique; (2) to determine environmental control and inducibility of hardiness at different parts of the first seasonal growth cycle; and (3) to examine the independence of acclimation, loss of hardiness and flushing in climatically "split" plants. Genetically identical material from hardy and nonhardy branches of the split plants was then used in conductometric and calorimetric studies of (4) the freezing process, and (5) the energy of tissue water, to see if adaptive changes in these could account for the induced hardiness differences.

The measurement technique involved freezing samples of excised needles under controlled conditions to various temperatures. Injury was estimated as the degree of browning after 7 days, and hardiness defined as the temperature causing 50% injury. Injury to excised needles was correlated with injury to attached needles, which was in turn broadly related to ultimate survival of whole plants frozen at the same temperature. Random error and possible bias in hardiness estimates both increased in hardier populations. Ten percent hardiness differences (e.g., two degrees in twenty) between populations could be detected with fifteen-plant samples; changes of 1°C could be followed during the course of treatment of an individual seedling.

Controlled environment studies in the first growth cycle showed that germinants (1 week) were unable to attain any freezing tolerance under 8-hr days at 2°C even after 9 weeks, but were killed whenever ice formed. However, seedlings older than 3 weeks (1 to 2 cm of epicotyl) could develop true hardiness under the influence of either short days (less effective) or low temperatures (above 0°C), independently of lignification, bud setting or entry into rest. Ability to acclimate increased gradually with age, and was inversely related to growth and maturation, apparently because the latter processes had higher temperature optima. Photoperiod affected growth and bud formation only above about 15°C, but influenced hardiness at 1°C with a longer inductive daylength at low than at high light intensities (12 and 8 hours respectively).

Interruption of the long inductive dark period with 15 min of red light (650 nm) caused a decrease in hardiness and bud set, and an increase in growth. Far-red interruptions (750 nm) alone had no effect, but enhanced the red light effect when applied immediately afterwards. Night frosts (-7°C) caused significant dehydration, and rapidly increased hardiness, only if both the warm short-day, and chilling treatments had been given in sequence first, and the daily supply of light continued. These results are in general agreement with the hypothesis that cold acclimation takes place in three physiologically distinct stages under natural conditions.

Studies on 3-year-old seedlings were carried out by exposing each branch of a forked plant to a different temperature (2° or 20°C) but similar light conditions for periods of one to five months. The chilling stimulus for breaking rest and inducing hardiness was confined to the chilled branch, but the warm branch apparently transmitted a factor which prevented full hardening in the chilled one. A factor moving in the same direction also promoted flushing in branches chilled only at night from December to June (and receiving greenhouse temperatures and natural photoperiods by day). This was not replaceable by a single injection of gibberellic acid. Factors from the expanding shoot caused loss of short-day-induced hardiness in previous year's foliage and stimulated cambial division. Chilling at night prevented the dehardening but did not prevent cambial activity. The dehardening factor was translocated to an opposite branch whereas movement of cambium stimulator was strictly basipetal. These results suggest that promoter-inhibitor levels controlling dormancy are independently regulated, and that a two-stage dehardening process might protect against premature loss of hardiness in nature.

The progress of freezing in needles of the hardy/nonhardy branch pairs was recorded simultaneously by differential thermal analysis and the conductance of low voltage direct electric current. The results of both methods exhibited the same major patterns. Freezing in immature leaves was nonequilibrium and intracellular. Freezing in needles cold-acclimated under short days was an equilibrium process preceded by a

short non-equilibrium freezing of the free intercellular water fraction. This pattern did not change in leaves more deeply cold-acclimated by low temperatures. Thawing in mature needles was characterized by a greater proportion of ice (than during freezing) at all temperatures, with indications that not all the original cell water was reabsorbed. Freezing records are interpreted as showing that the cell membrane became more permeable to ions after injurious slow freezing but retained its essential integrity, whereas rapid freezing caused immediate membrane damage. No features of the freezing or thawing curves of first or subsequent freeze-thaw cycles were useful as predictors of injury to needles by slow freezing.

Energy of water in hardy/nonhardy needle pairs was compared by two methods. Heats of vapourization (ΔH_v) of weighed increments of water, removed from excised needles under vacuum, were estimated from the calibrated vapourization endotherms recorded on a differential thermal analyser. In the second method, needle water contents were measured gravimetrically after equilibration with lithium chloride solutions of known desiccating energy. It was found that ΔH_v, a proposed measure of water binding near surfaces, increased as the proportion of water remaining in the leaf decreased. For each increment removed, ΔH_v was significantly higher in hardy needles, notwithstanding various possible sources of error. Hardy needles also retained more water non-osmotically (than nonhardy needles) after equilibration with LiCl isopeistic with their frost-killing temperature. The date suggest

that avoidance of dehydration, principally by non-osmotic lowering of cell water potential, can account for almost half of the 25 centigrade degree difference in hardiness between branches.

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ACKNOWLEDGEMENTS

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INTRODUCTION

The ability of plants to withstand freezing has been the subject of research for over a century, comprising an estimated[1] 6,000 published papers. Several good reviews are available[2] and no general review is attempted here. The present work was begun in 1968 to extend the knowledge to small (containerized) conifer seedlings being used in reforestation programmes. The research is presented in five more or less self-contained sections according to the approximate progression: measurement of hardiness → environmental control → aspects of internal control → freezing patterns and characteristics of tissue water. Each section contains its own specific literature review, discussion and abstract. The principle findings and conclusions of the study as a whole are summarized in the preceeding abstract.

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CHAPTER 1

AN EXCISED-NEEDLE FREEZING TEST OF COLD HARDINESS IN DOUGLAS-FIR

ABSTRACT

A freezing test is described which is suitable for measuring hardiness changes in small conifer seedlings during physiological studies. The method involves freezing samples of excised needles, under controlled conditions, to various temperatures. Injury is estimated as the degree of browning after 7 days. Hardiness is defined as the temperature causing 50% injury.

Injury to excised needles was correlated with injury to attached needles, which was in turn broadly related to ultimate survival of whole plants frozen at the same temperature. Dexter's electrolytic method provided a less sensitive measure of injury, but both visual and electrolytic estimates were significantly correlated. Random error and possible bias in hardiness estimates both increased in hardier populations. Ten percent hardiness differences (e.g., two degrees in twenty) between populations could be detected with fifteen-plant samples; changes of 1°C could be followed during the course of treatment of an individual seedling.

INTRODUCTION

During research into the physiology of cold acclimation in conifer seedlings it was found desirable to have a standard measure of hardiness. Ideally, this needed to be (1) non-destructive, so that changes in a single plant could be followed; (2) expressible as a "killing temperature" so that the information could be related directly to field conditions and compared with the work of other investigators using other species; (3) reproducible and reasonably quick.

These requirements are somewhat conflicting. To obtain measurements of lethal temperatures by freezing whole plants is destructive; it requires excessive replication and cumbersome apparatus; and it does not simulate natural conditions if roots are frozen as well. Completely non-destructive methods (5, 6, 12, 16, 21, 23) are either too inaccurate, even for field use, or else make questionable assumptions about the process being measured, so that freezing tests are ultimately necessary. Excised parts have therefore been used (8, 9, 15, 17), but these undergo chemical changes (7) that might affect development of injury, and may exhibit an atypical freezing pattern (3, 10, 13). Furthermore, the excised tissue or organ may not be critical to survival of the whole plant (1, 14, 18). The following is a brief description of the partly destructive method adopted using small excised-needle samples, the variation

in freezing injury (assessed by alternative criteria) thus obtained, and the precision with which survival of attached foliage and whole plants is predicted.

MATERIALS AND METHODS

Plants

Pseudotsuga menziesii (Mirb.) Franco seedlings less than one year old and from one of two southern Vancouver Island provenances provided most of the data. The two provenances were Mt. Prevost, 500 m (lat. 48°50' x long. 123°40') and Mt. Benson, (49°09' x 124°04'). Data given below refer to the Mt. Prevost provenance unless otherwise stated. Plants were raised in 2 x 11 cm pots under favourable conditions in a greenhouse or under partial shade outdoors, with regular watering and nutrient application. They were subjected to various cold-acclimation treatments, specified below, to produce a range of hardiness.

Freezing of Whole Plants

The aim of all tests was to measure low temperature hardiness, not tolerance of abnormally rapid freezing or thawing rates (2), which has a different physiological basis and is of limited practical concern. Whole plants were frozen in an insulated box with internal fan in a -25°C cold room at 4°C/hr. Subsequently a maximum safe cooling rate of 7°C/hr was established, and temperature was positively controlled by small fan-heaters inside a commercial freezer, the heaters being

linked to a temperature programmer. Plants in the insulated box warmed at 10°C/hr, and the others at 20°C/hr after attainment of the minimum temperature (injury was shown to be independent of warming rates less than 40°C/hr). Temperatures monitored by thermocouples placed in various parts of the foliage differed by a maximum of 1.5°C in the box, and 1.0°C in the freezer. Plant positioning was randomized to avoid bias. Pots were soaked with water for several hours before freezing to equalize sap tension, turgor, soil freezing, atmospheric humidity and therefore to a certain extent, supercooling.

Excised-Needle Freezing Test

Samples consisted of 7 to 10 needles removed from equally spaced positions down the stem so as to transect age variation. For each plant, three or more such samples were taken and each placed base-down in a labeled, clean 4.5 ml vial containing a few drops of distilled water. Samples at this stage could be conveniently stored overnight at 1°C without significant effect. The number of samples per plant depended on abundance of needles and foreknowledge of the approximate killing temperature; careful removal of a large proportion of the needles affected neither acclimation of the remainder nor subsequent bud burst. The three or more unstoppered sample vials from each plant were stood in separate racks which were placed beneath fans in the programmed freezer. Replicate sample racks were removed

from the freezer (fans off) at successively lower 3°C temperature intervals chosen to include the lethal temperature. They were allowed to thaw in precooled polystyrene containers placed in a cold room at 1°C. Temperature was recorded continuously, by thermocouples in various vials, on a multi-channel strip chart recorder. The range of temperature at any given time during cooling, among large numbers of samples, seldom exceeded 0.5°C if vials were spaced to permit free air flow and raised above the freezer floor. Water in vials was seeded by a shower of fine ice crystals at -3°C to equalize supercooling in the leaves.

Assessment of Injury

Injury to foliage was estimated visually and, in some cases, by both visual and electrolytic methods (the latter method is described below). Visible injury to foliage of whole plants, after a 4-day recovery period under a 16-hour photoperiod at 21°C and 1000 ft-c was given a value of 0 to 10 based on the fraction of needles completely brown or more than one-half discoloured. Visible injury to each needle of excised-needle samples was assessed as number of tenths discoloured or brown after a 7-day dark incubation period at 21°C in the stoppered vials. The total for 10 needles was expressed as a percentage.

The electrolytic method records the ease with which electrolytes diffuse from the cells into surrounding water as a result of injury to the cell membrane, and was originally

developed by Dexter et al. (4). In dilute solutions at constant temperature conductivity is proportional to ionic concentration. The ratio of conductivity of the bathing solution after freezing to that after complete killing (the relative conductivity, RC) thus quantifies freezing injury independently of initial tissue electrolyte concentration, mass of tissue or volume of bathing solution. For electrolytic determination of foliar injury to whole plants, a vertical transect sample of 7 to 10 needles was taken from each, with clean forceps immediately after thawing. Samples were shaken with 3ml distilled water (conductivity 3 ± 0.5 μmho) in clean, stoppered vials for 12 hr at room temperature, and measured with the CDC 104 conductivity cell of a Radiometer, Copenhagen, conductivity meter at 21 ± 0.5°C. Tissue was then killed by immersing vials in a water bath at 95°C (under pressure to prevent evaporation) or in liquid nitrogen, for 5 min (both methods are compared below). Final conductivities were measured after shaking for a further 6 hr. For determinations of injury in frozen excised-needle samples, vials containing the needles were made up to 3 ml with distilled water and the above procedure followed.

Ultimate recovery of whole plants in a controlled environment favourable to growth was recorded after 3 months. They were classified as recovered (new growth), indeterminate (partly green but quiescent), or dead.

RESULTS & DISCUSSION

Comparison of Injury Criteria

Figure 1 shows that both electrolytic and visual measures record an increasing degree of injury with lower freezing temperature. But the electrolytic measure has both a smaller range, and a greater variance among individual plants. The use of liquid N rather than hot water for killing did not alter this variance significantly, contrary to Sukumaran and Weiser's findings (19). The following changes in procedure also failed to materially reduce variance in RC: lengthening or decreasing elution time, using purer distilled water, surface washing leaves, and increasing the number of needles per sample. This relative insensitivity of RC was confirmed during other experiments, and suggests that the cell membrane is perhaps not the primary site of injury in Douglas-fir. A similar result has been reported for apple shoots (11) but not for Scots pine (2). It was also noticed during the present investigation that electrolytic measurement became more sensitive if an incubation period was allowed, as in the procedure for visual estimation. However, the electrolytic method, which is more laborious, then loses its practical advantage of providing earlier results.

Although visual estimation is more subjective, a second assessment of one experiment after re-randomization scored whole plants at their original value with occasional 1-unit deviations;

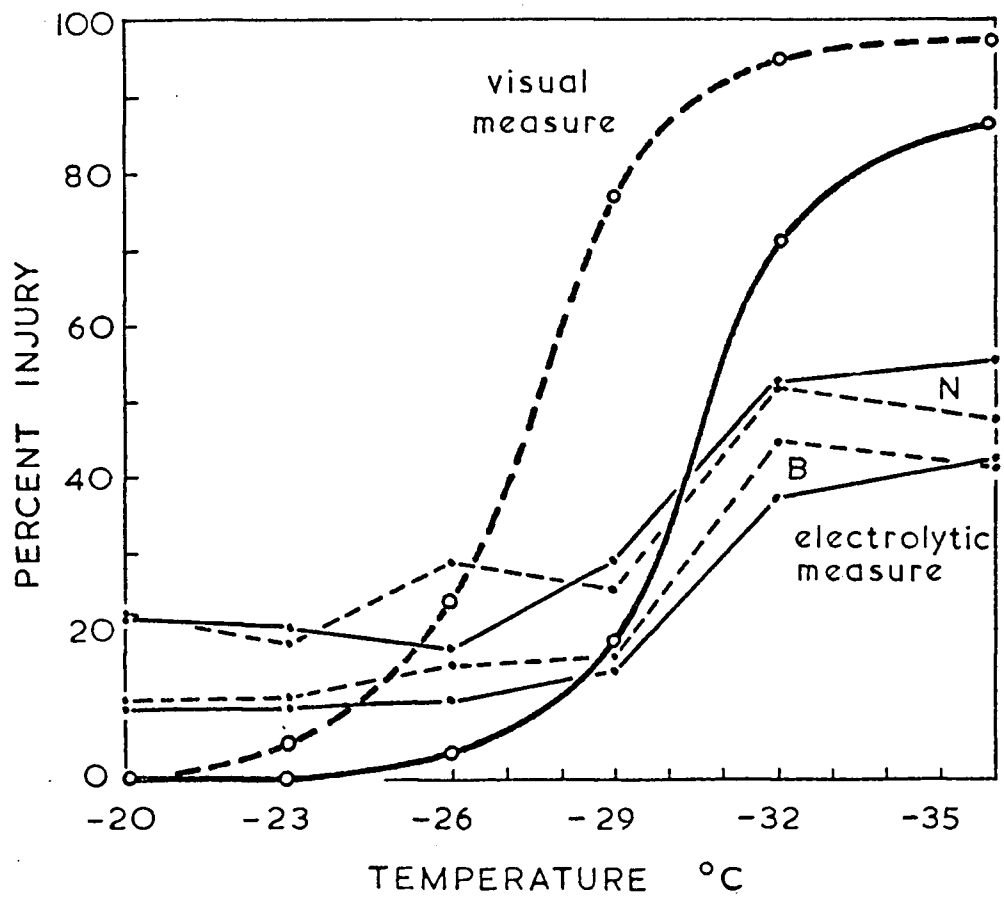

Fig. 1. Sensitivity comparison of electrolytic and visual measures of freezing injury to excised needles. Visual estimates of browning are fittable by smooth curves and readily distinguished between two different groups differing slightly in hardiness (solid line is relatively hardy). Conductivity of bathing solution, expressed as a percentage of that after killing with liquid nitrogen (N) or boiling water (B), has a smaller range and does not clearly differentiate hardiness groups. Six plants comprised a hardiness group. Each point is the mean of 10-needle samples from the six plants.

excised samples were within 5%, and conclusions were unchanged. The amount of possible bias is discussed below.

Correlation of mean electrolytic and visual injury values from a series of acclimation treatments was high (Fig. 2), but for individual plants correlation was low due mainly to the variance in RC. Exceptionally good individual correlation, however, was found for an interior provenance (Fig. 3). Zehnder and Lanphear (22) reported a 0.97 correlation for leaves of Japanese yew.

Injury by either visual or electrolytic measures was related to final survival of whole plants (Fig. 2). The average level of visible foliar injury corresponding to a 50% survival of whole plants was only about 20% in Figure 2, due to root damage under the artificial conditions used to freeze whole plants in these experiments, and due to an insufficiency of mature lateral buds to resume growth. Under natural conditions roots are protected from the air temperature minima, and therefore survivals corresponding to a given level of foliar injury would be higher. Alden (1) found that the leaves of this species were the parts actually limiting winter survival. Consequently, in the absence of better data, the 50% level of visible foliar injury was considered to define the lethal temperature. This is also in accordance with other studies (17, 19).

Figure 4 shows that visible injury to attached foliage

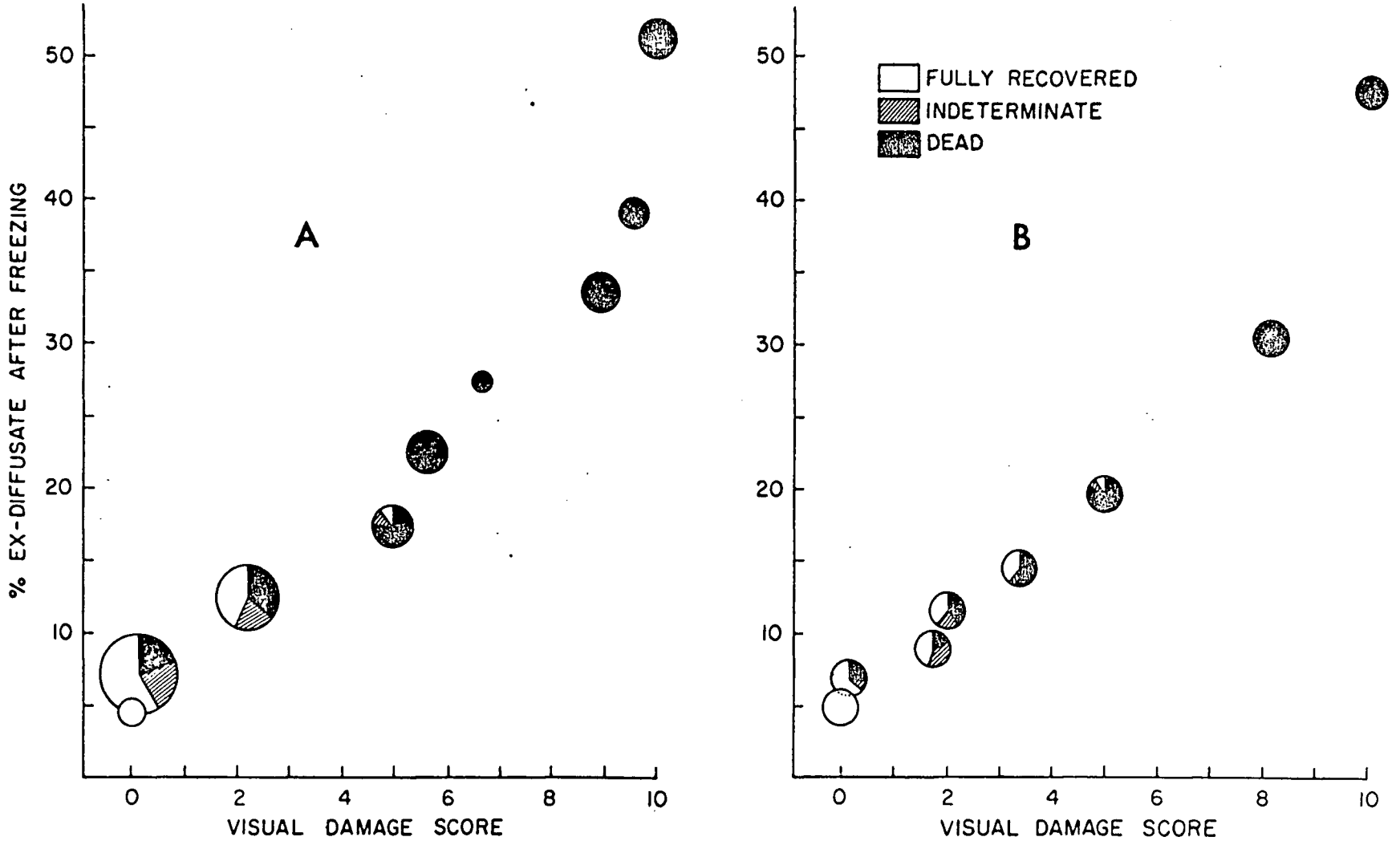

Fig. 2. Correlation between mean electrolytic and visual values for freezing injury, and ultimate survival of whole plants. Data are from a total of 90 6-month-old plants subjected to a range of acclimation treatments and freezing temperatures. Plants were frozen in insulated boxes in a cold room and needle samples excised for conductivity measurements afterwards. The visual injury score refers to attached foliage. In A, circles represent groups defined by 5% intervals across the range of conductivity values, the area being proportional to the number in the group (minimum, 2). In B, circles represent groups of 11 plants taken in order across this range.

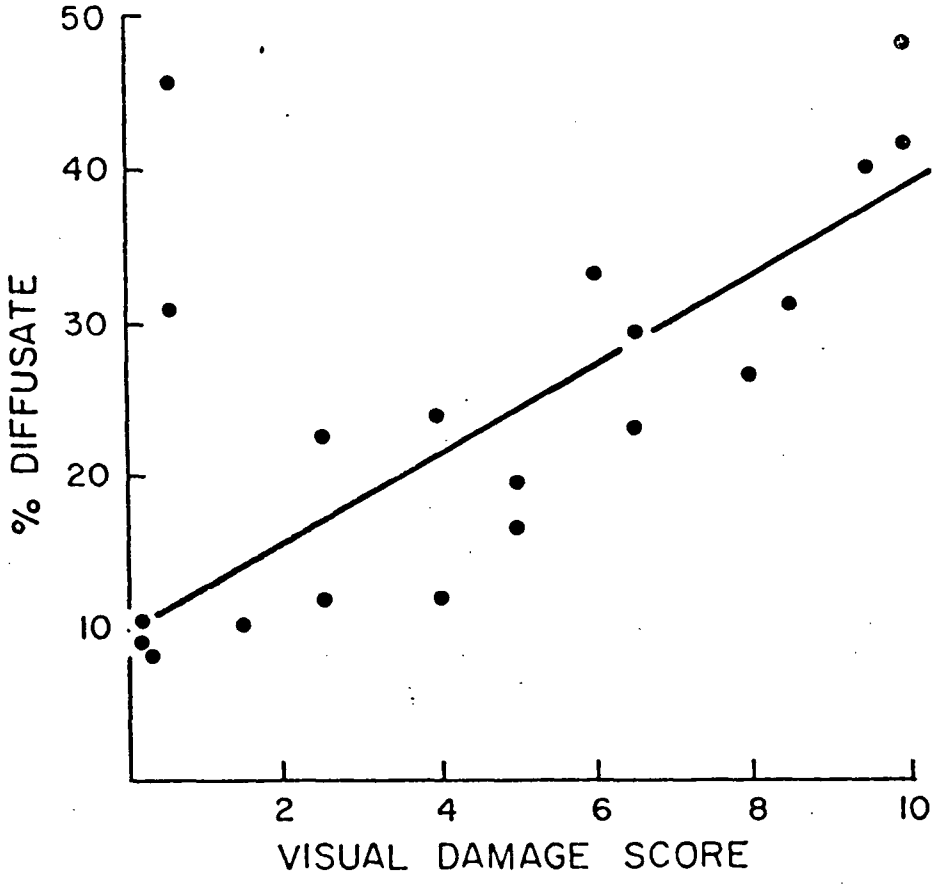

Fig. 3. Correlation between individual electrolytic and visual injury values for freezing injury to whole plants. Data are from an interior B.C. provenance (800 m, Prince George, B.C.) subjected to a range of acclimation and freezing treatments. Each point represents one plant, assessed as described in Figure 2. The two uppermost points close to the ordinate have been omitted from the calculated regression: $y=10.30+2.94x$; $R=0.86$.

could, within the limits of error, be measured directly as that inflicted upon excised samples under the described conditions (regression coefficient $\triangleq 1$). Subsequent exothermal measurements of the freezing process (20) showed that the rate and pattern of ice formation was also similar in excised needles providing that an external contact with water permitted entry of ice through the vascular system as in the present case. Agreement between injury to excised shoots and whole plants of Scots pine has been reported (2).

The foregoing data show that measurements of hardiness of excised needles are suitable not only for specific comparisons of foliar hardiness under laboratory conditions, but also have meaning in terms of some intermediate survival in populations of intact plants.

Uncontrolled Variation in Visible Injury

Anatomical variation in injury to a single needle has been described by Alden (1) with reference to an 8-point scale. The clarity with which browning could be classified macroscopically on the present 10-unit scale varied according to the provenance, age and history of treatment. In the best cases, the dividing line between brown and green was abrupt and transverse, demarcating an easily measurable length of dead tissue. In the worst cases, notably old needles, interior provenances, and deeply acclimated leaves, a general discoloration pervaded the entire

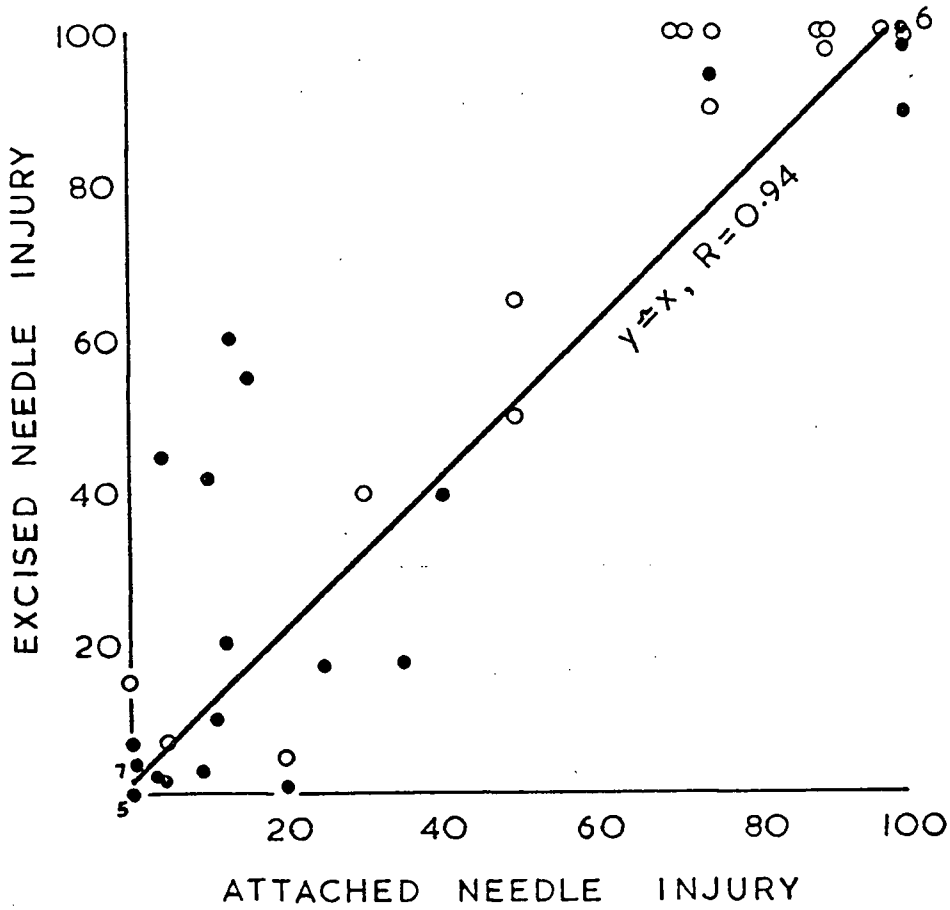

Fig. 4. Correlation between freezing injury to excised and attached needles. Each point represents one one-year-old plant. 10-needle samples, frozen before or after excision, were assessed for percent browning after 7 days. The Mt. Prevost provenance (●) and a less hardy coastal provenance of uncertain origin (0) had been subjected to a range of acclimation treatments. Digits indicate number of coincident points. R is the correlation coefficient. Intercept and slope coefficients do not differ significantly from zero and one respectively.

leaf. Injury could be estimated to 1 unit and 3 units respectively. A photographic record of groups classified to a 2-unit accuracy was used to standardize injury estimation in different experiments. This is shown in Figure 8 together with a photograph illustrating the variation in injury within and between whole plants at different levels of hardiness. In the worst and highly improbable case, a subjective 3-unit bias in the excised-needle injury estimates of highly hardy plants could lead to an error in mean lethal temperature of 4°C (Fig. 6). Normally, however, unidirectional 2-unit misclassifications of two or three needles at only some of the test temperatures would displace the lethal temperature of an individual plant by less than 0.5°C.

Variation within a plant is shown in Figure 5. Needles of intermediate age were hardiest; those at the tip least hardy. The inclusion of young needles in the transect sample was advantageous in affording some measurable injury even when selected test temperatures failed to include the lethal level.

Variation in injury curves between plants of uniform age, treatment and appearance is shown in relation to the mean curve B, in Figure 6. The individual injury curves tend to be parallel at low to moderate hardiness levels, so that hardiness of individuals can be reliably ranked. A 92% difference in injury at 11°C (between extreme curves of the B population) represented a difference in lethal temperature of 6°C. Mean of the interpolated lethal

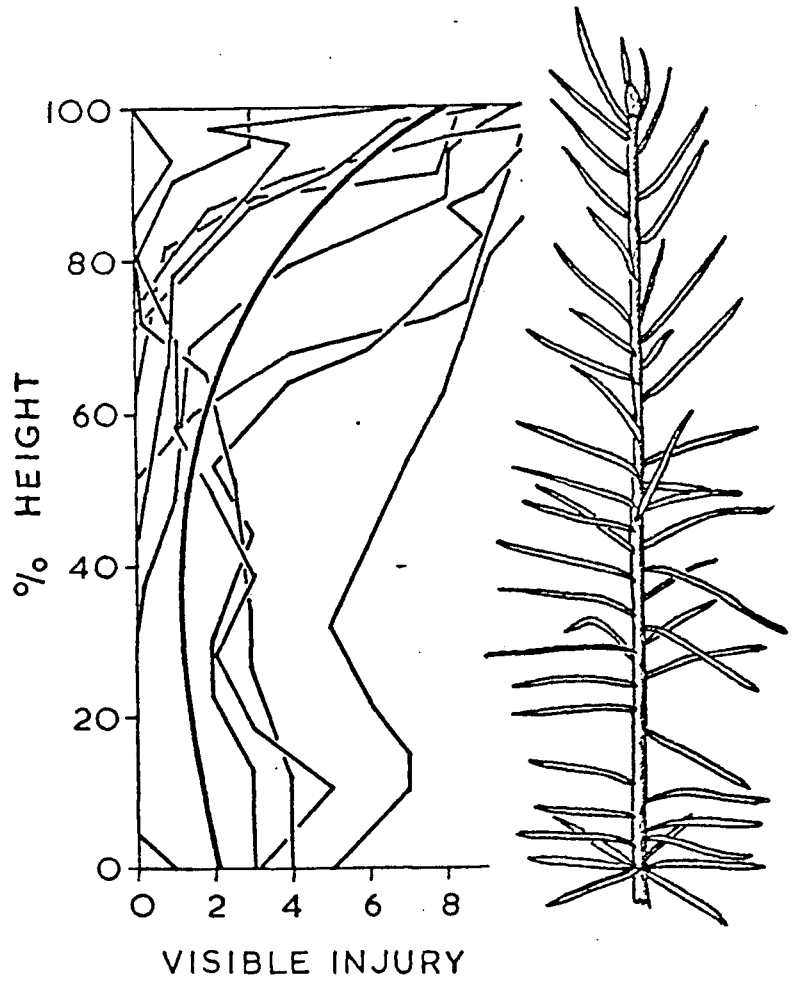

Fig. 5. Age variation of visible freezing injury within a plant. Bold curve is the mean of the individual curves from 10 6-month-old seedlings. Each point on an individual curve is the visible injury score to a single needle removed from that position on the stem 4 days after the whole plant had been uniformly frozen and thawed. Similar variation was exhibited by needles excised before freezing.

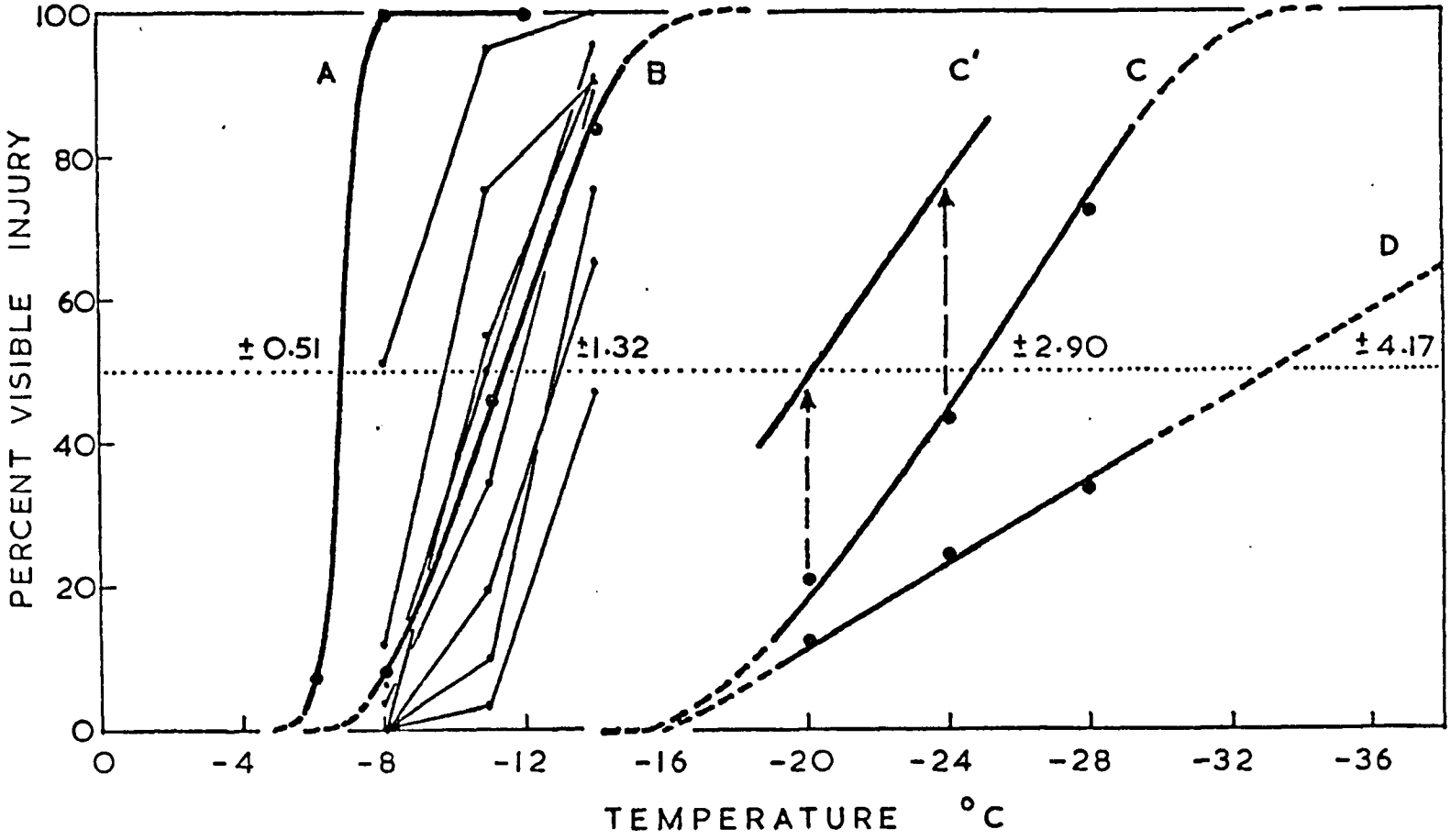

Fig. 6. Variation in visible freezing injury between plants, and at different hardiness levels. Plants were of the Mt. Benson provenance. Populations A, B, C and D were the result of increasingly effective acclimation treatments of similar duration. Some individual injury curves contributing to the mean curve (bold line) are shown for B. Each of the 3 points on an individual curve represents injury to a 10-needle excised sample. 95% confidence limits on the hardiness (defined as temperature corresponding to 50% injury) are shown for each population. The effect of a 3-unit overestimating bias on apparent hardiness is shown by C'.

temperatures was 11.2±1.3°C (0.05 probability level) in this example; lethal temperature from the mean curve was 11.3°C. The latter derivation usually differed by 0.2 to 1.0°C, and was used only when individual curves could not be reliably extrapolated.

The slope of the mean injury curve decreased with increasing hardiness, leading to greater possible bias errors (Fig. 6). Associated between-tree variation also increased (95% confidence limits are given), but remained relatively constant when expressed as a percentage of hardiness. This behaviour was exhibited by both coastal and interior provenances. The minimum size of sample needed to detect a given hardiness difference between populations therefore depends on the average hardiness level. An approximate rule is that 10% hardiness differences (e.g., 1 degree in 10, 3 in 30) between populations will be detectable with a sample of 15 plants from each.

Figure 7 shows differences of equally treated individuals from the same provenance with respect to time. Shape of acclimation curves was similar. Seedlings destined to become relatively hardy generally exhibited this advantage at an early stage of acclimation. The time of sampling to detect individual differences, for example in genetic studies, would therefore not be crucial.

CONCLUSIONS

Freezing excised needles, followed by visual estimation of degree of browning as described, provides a valid, absolute

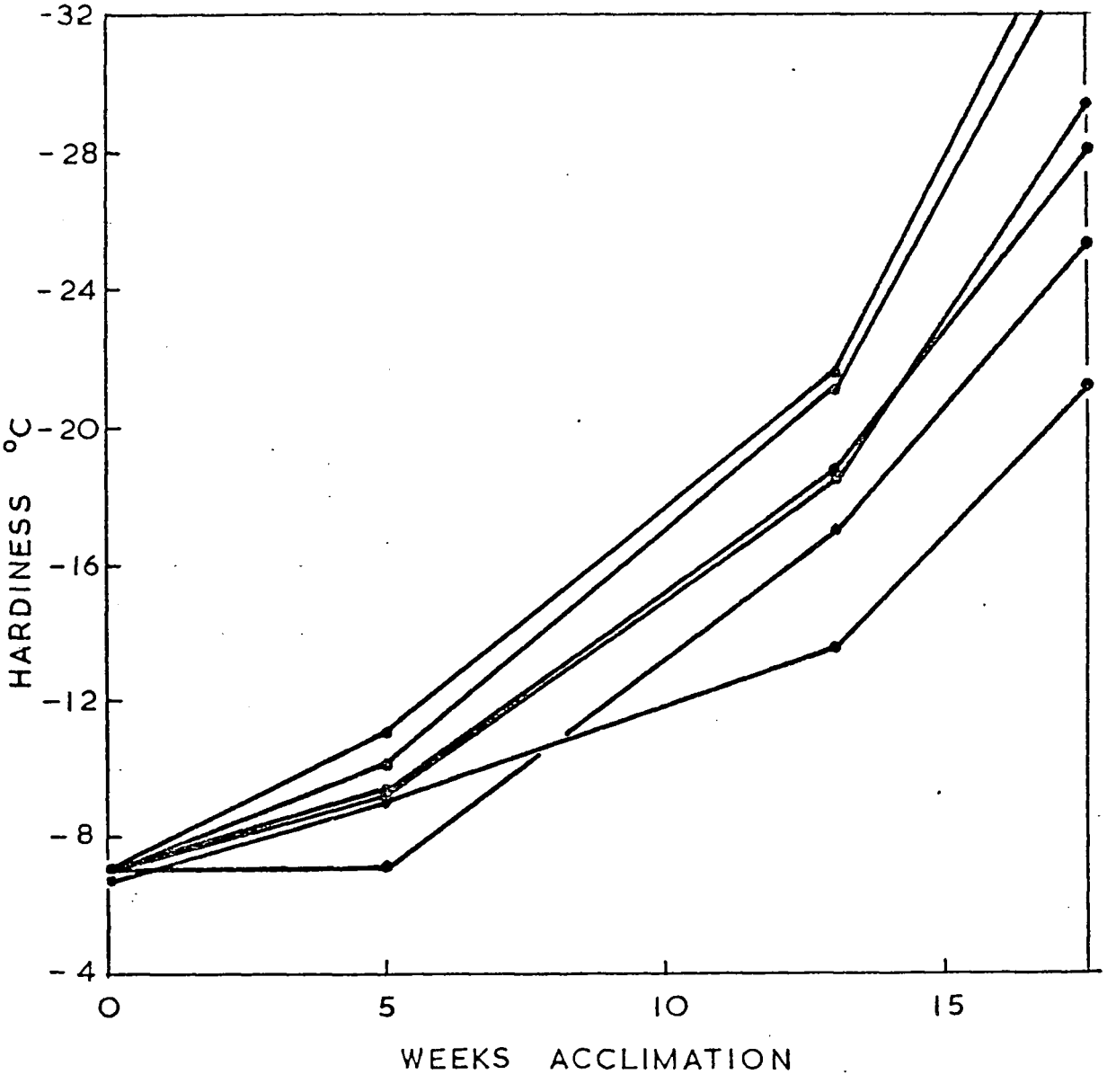

Fig. 7. Variation in acclimation rates of individual seedlings, Mt. Benson provenance. Hardiness was assessed visually on excised-needle samples. Points on a curve represent successive determinations on the same plant during the course of progressively more severe acclimation treatment. Superiority tends to appear early and be maintained.

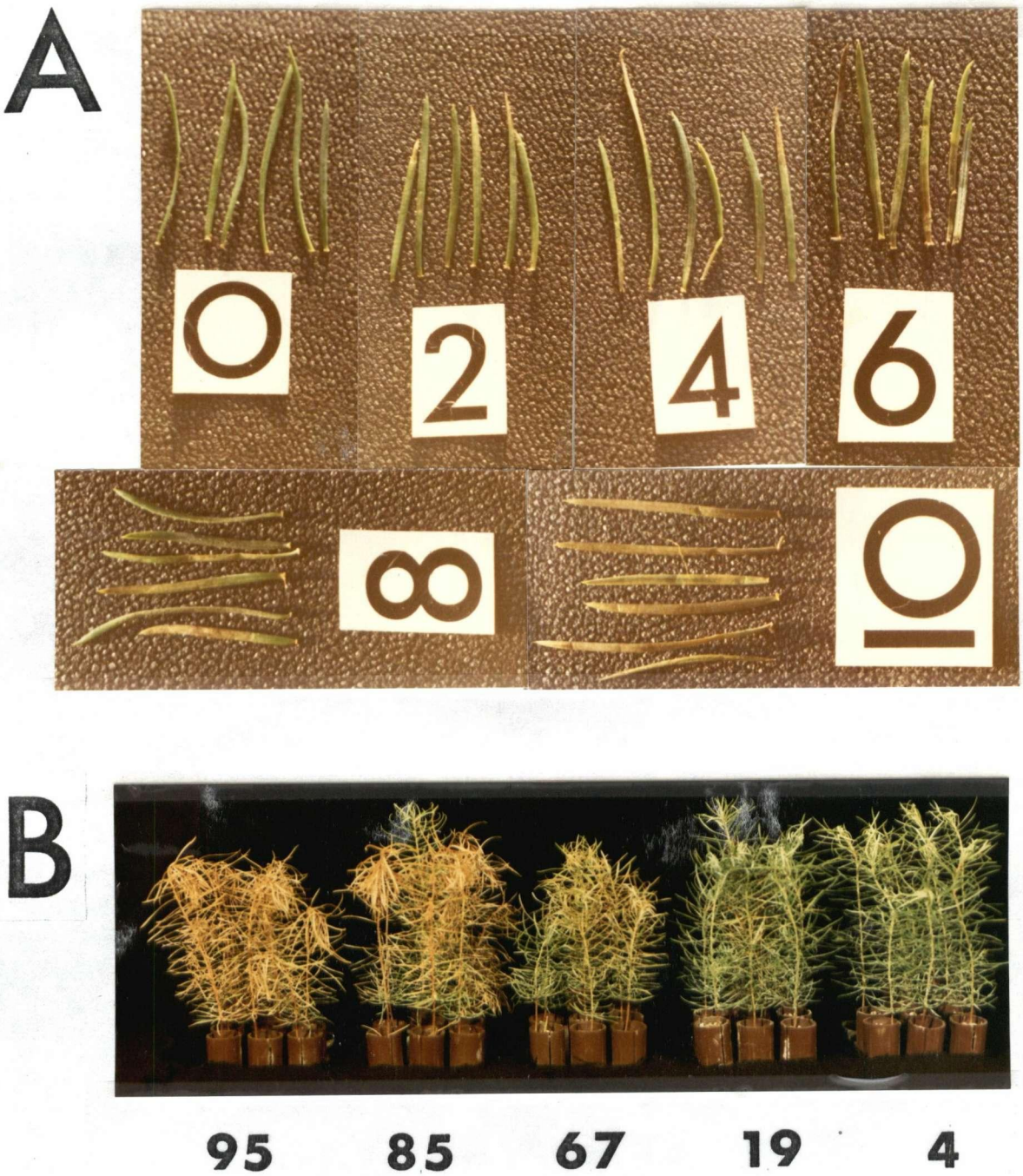

Fig. 8. Classification of visible injury to excised needles and to foliage of whole plants. A illustrates classification of individual needles 7 days after freezing in vials by the standard procedure (see materials and methods). Each needle in a group had the number of tenths of brown tissue indicated by the figure beneath. For comparison, B shows actual injury to 5 groups of 8 3-month-old seedlings 4 days after freezing at -8°C. Groups were acclimated at 1°C under an 8-hr photoperiod for (from left to right) 0, 2, 3, 4 and 5 weeks. In this case the average of the 8 whole-plant injury scores (see materials and methods) is given as a percentage beneath each group.

rather than comparative measure of foliar hardiness for physiological studies. Variation in test stresses is minimized by accurate control of cooling rates (any of several published or commercial methods could be used), prior water storage and early ice seeding. Unwanted variation in visible injury is reduced by sampling technique, comparison with defined standards, repeated use of the same individuals and selection of appropriate sample size. Greater objectivity and earlier results (desirable for nursery work) could be obtained by the electrolytic method at the expense of heavier sampling and more work per sample. Estimates are applicable specifically to attached foliage; they are related to winter survival of whole plants, and they are reproducible.

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CHAPTER 2

ENVIRONMENTAL CONTROL OF COLD ACCLIMATION IN DOUGLAS-FIR DURING GERMINATION, ACTIVE GROWTH AND REST

ABSTRACT

Controlled environment experiments were conducted on Pseudotsuga menziesii (Mirb.) Franco seedlings during their first year. Hardiness of foliage was assessed by visually estimating injury after freezing tests.

Germinants (1 week) were unable to attain any freezing tolerance under 8-hr days at 2°C even after 9 weeks, but were killed whenever ice formed. Their ability to supercool increased by 5°C during this treatment. However, seedlings older than 3 weeks (1 to 2 cm of epicotyl) could develop true hardiness under the influence of either short days (less effective) or low positive temperatures, independently of lignification, bud setting or entry into rest. Ability to acclimate increased gradually with age, and was inversely related to growth and maturation, apparently because the latter processes had higher temperature optima. Photoperiod affected growth and bud formation only above about 15°C, but influenced hardiness at 1°C with a longer inductive day-length at low than at high light intensities (12 and 8 hours respectively).

Interruption of the long inductive dark period with 15 min of red light (650 nm) caused a decrease in hardiness and bud set, and an increase in growth. Far-red interruptions (750 nm) alone had no effect, but enhanced the red light effect when applied immediately afterwards. Night frosts (-7°C) caused significant

dehydration, and rapidly increased hardiness, only if both the warm short-day, and chilling "stages" had been supplied first, and the daily supply of light continued.

INTRODUCTION

The environmental control of cold acclimation in coniferous evergreens in relation to their early growth and development has received little study in spite of its obvious relevance to forest nursery work. Most investigations have been carried out at the end of a growing season on broadleaved woody species which, unlike conifers, cannot acclimate substantially during their period of active growth, nor in most cases, attain maximum hardiness while still bearing functional leaves. Several workers have found that two or three sequential stages must be followed for maximum and most efficient acclimation in the broadleaved group (9, 16, 22). These stages have been summarized by Weiser (24) as occurring in response to short days (and entry into winter dormancy or "rest"), cool temperatures (and leaf abscission), and prolonged sub-zero temperatures (lower than -30°C). Their independence of each other and of associated phenology, however, appears to vary considerably between species (7, 8, 10).

In conifers, this pattern of change has not been described, although there is scattered evidence for some sequential processes. The separate effectiveness of short days and low temperatures in inducing hardiness has been widely reported (5, 13, 26), but maximum hardiness has not been shown to depend on the initial exposure to short days when long acclimation periods are considered; nor has the role of rest, either in acclimation or loss of hardiness, been

established. In fact, preliminary experiments in the present study indicated that substantial acclimation can occur during the temporary suspension of active growth at low temperatures. Cabbages apparently acclimate best while continuing growth (2), which indicates that, in some species at least, the two processes are not biochemically opposed. Scheumann and Bortitz (18) observed a rapid acclimation response to temperatures a few degrees below the freezing point as opposed to acclimating temperatures just above zero, thus indicating a further deviation from the broad-leaved model. Others have failed to detect a sub-freezing temperature effect (5, 26). The following investigations were begun in 1968 to more closely define the environment, growth and hardiness relationships in conifer seedlings during the first seasonal cycle from germination to winter rest.

GENERAL METHODS

Plants

A total of five experiments was conducted on *Pseudotsuga menziesii* (Mirb.) Franco seedlings during or immediately after their first season's growth. Four British Columbia provenances were used: (1) southern Vancouver Island, 500 m (Mt. Prevost, lat. 48°52' x long. 123°45'), (2) southern Vancouver Island, 800 m (Mt. Benson, 49°09' x 124°04'), (3) Prince George, 800 m (54°15' x 122°50') and (4) Chilliwack, 1050 m (Mt. Thurston, 49°07' x 121°04'). The lower elevation Vancouver Island provenance was used in all

experiments, and descriptions refer to this unless otherwise indicated. Plants were raised in 2 x 11 cm pots in peat-sand or peat-vermiculite mixtures under favourable conditions, either in a greenhouse or under partial shade outdoors. They were fertilized weekly with a complete nutrient solution. In all experiments, controls and treatments at warm temperatures (≒15°C) continued to receive fertilizers, though less frequently, in order to maintain health. Subsequently it was shown (21) that hardiness was not significantly affected by this extra feeding.

Evaluation of Hardiness

Hardiness was measured either by freezing whole plants in an insulated box in a cold room (subsequently a programmed freezer was used), or by freezing samples of 7 to 10 excised needles in vials according to the methods previously described (20). Cooling rates did not exceed 7°C/hr, nor warming rates 20°C/hr, so that in all cases injury depended only on the lowest temperature reached. Injury was assessed either as degree of browning of foliage on a 10-unit scale, or as the fraction of electrolyte leached from the tissue according to the method originally developed by Dexter *et al* (3).

Where excised-needle samples were frozen, determinations were repeated at successively lower 3°C intervals on the same group of plants and a "lethal temperature", corresponding to 50% visible injury, was interpolated. This provided an absolute rather than comparative measure of hardiness related to ultimate survival of whole plants (20). Lethal temperature was analysed by analysis of variance and means were tested by Duncan's Multiple Range Test. Visual injury scores (at a single freezing temperature), and percentage conductivity data, were first "normalized" by arcsine transformation.

EXPERIMENTS AND RESULTS

Experiment 1 - Growth, Temperature and Photoperiod

This was carried out between July and November, 1968, in three cold rooms maintained at 1, 6 and 11°C (±1.3°C). In each cold room were three photoperiod treatments (8, 12 and 16 hr) at an incandescent light intensity of 100 ft-c. Temperatures during the light period exceeded the cold room temperature by 1.5°C. Six plants from each of Mt. Prevost and Prince George provenances were placed under each of these nine treatment combinations 6, 11, and 15 weeks after late-May germination. Control groups were placed in 75% shade outdoors at the same time, and for the earliest treatment an additional control was placed in a 12-hr, 21-15°C day-night, controlled environment.

Height, number of lateral buds and size of terminal bud (if present), were recorded before and after the 8-week treatment. Relative hardiness was measured by freezing whole plants at one or two temperatures and visually estimating injury after 4 days as described previously (20).

Relative hardiness is shown in Figure 1. There were substantial (and statistically significant) differences in hardiness, for both interior and coastal provenances, associated with temperature, photoperiod and the date on which treatments were begun. Even plants at an early stage of active growth (2 cm of epicotyl) had acclimated under short days and chilling to a hardiness level below -8°C. Acclimation under 12-hr days was greatest at all stages of development at the relatively low light intensity of this experiment. This photoperiod effect was relatively unimportant at low temperatures; it operated both on plants in active growth and those with buds set and in rest. Under the cool temperature (≈12°C), and at light intensity around the photosynthetic compensation point, photoperiod did not influence bud development or extension growth (data not shown). Growth and maturation were dependent only on temperature; both decreased under the low temperatures which favoured acclimation (Fig. 2).

Outdoor controls (which form the basis for percentage values in Figure 2) underwent normal completion of growth, lignification and bud development between the time of beginning first treatments

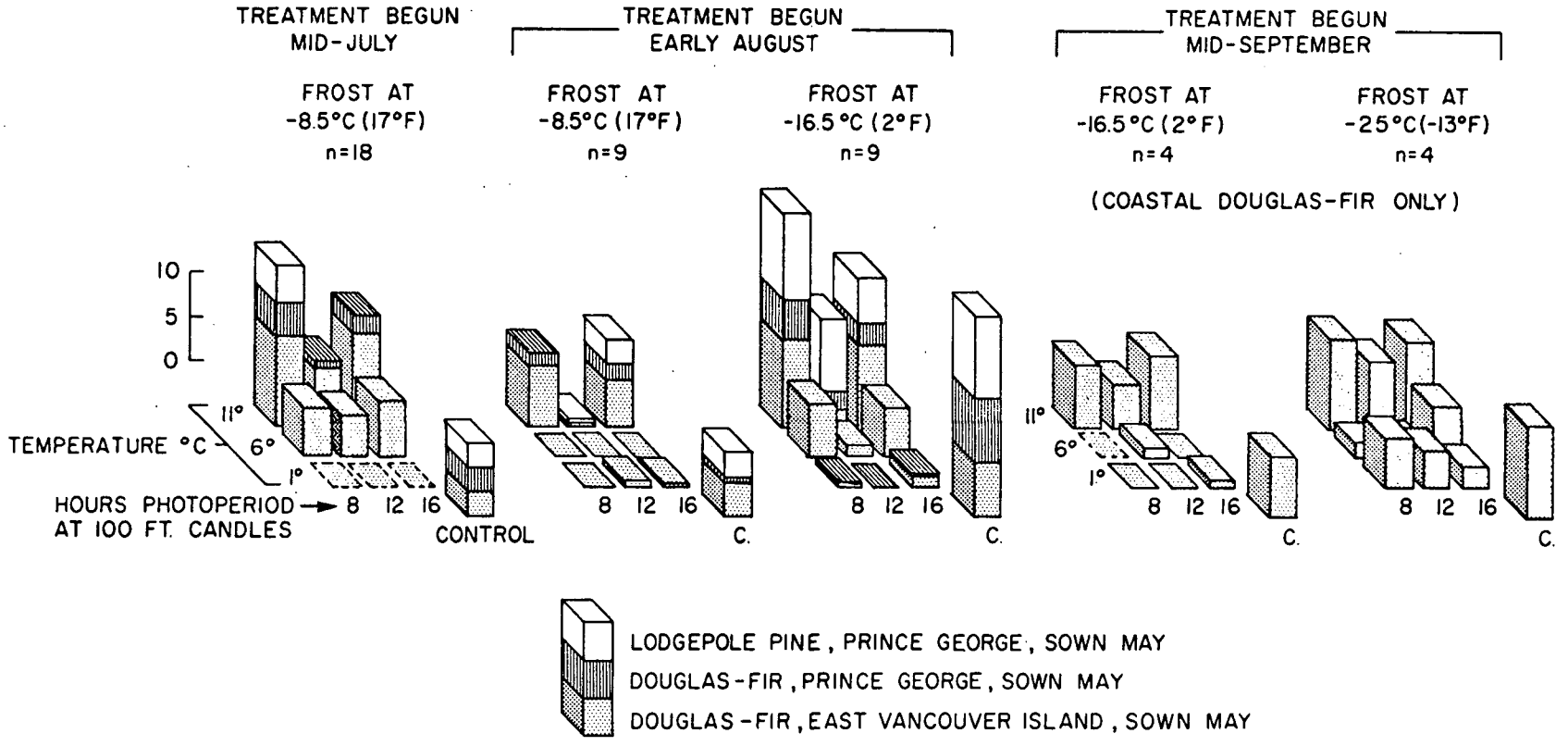

Fig. 1. Injury to seedlings after 8 weeks' acclimation under low light intensity (Experiment 1). Injury to whole plants was scored visually between 0 (undamaged) and 10 (completely killed) 4 days after freezing to the indicated temperature. The Mt. Prevost provenance occurs throughout all treatments. Pinus contorta Dougl. is also shown for comparison in July and August treatments. Each column is the mean of n seedlings, n/3 of each species or provenance.

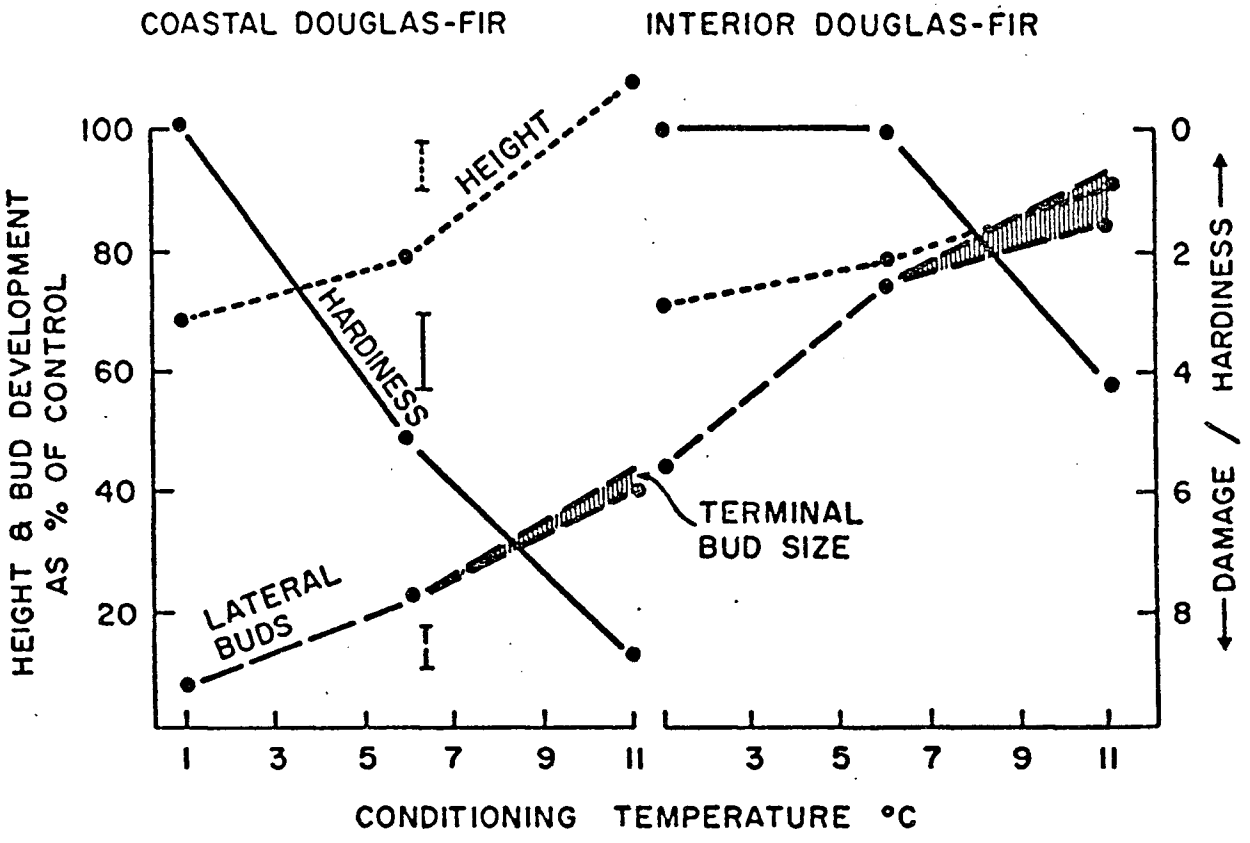

Fig. 2. Relationship between development and hardiness in coastal (Mt. Prevost) and interior provenances (Experiment 1). Least significant difference at 5% level is indicated, assuming common variance among provenances.

(July) and ending the last (mid-November). They acclimated gradually during this period, particularly the interior provenance which also began maturation earlier (Fig. 2) in the cold rooms regardless of photoperiod. The data illustrated in Figure 1 do not show this acclimation in the case of the coastal provenance due to accidental loss of replicates, but subsequent measurements confirmed it.

Experiment 2 - Acclimation of Germinants

Two types of seedlings were investigated in this experiment. Seedlings 1 to 2 weeks after germination possessed only hypocotyl cotyledons and a tuft of new needles; seedlings 2 to 3 weeks after germination had developed at least a centimeter of stem above the cotyledons and were physiologically independent of them. Seedlings of both types were given a 10-hr photoperiod with 300 ft-c mixed fluorescent and incandescent light, in a cold room at $1 \pm 1.5°C$. Samples were removed at intervals of 1, 2, 3, 4 and 9 weeks. They were tested in a freezing chamber designed to freeze only the above-ground parts, and to permit a continuous record to be made of electrical conductivity of stem and leaf tissue during freezing (Fig. 3). The purpose of this arrangement was to determine the temperature and approximate rate of ice formation in the tissue in accordance with Olien's method (14) which assumes that the amount of liquid intercellular water is proportional to the current flowing at low voltages. Gold-plated copper electrodes were attached to stems,

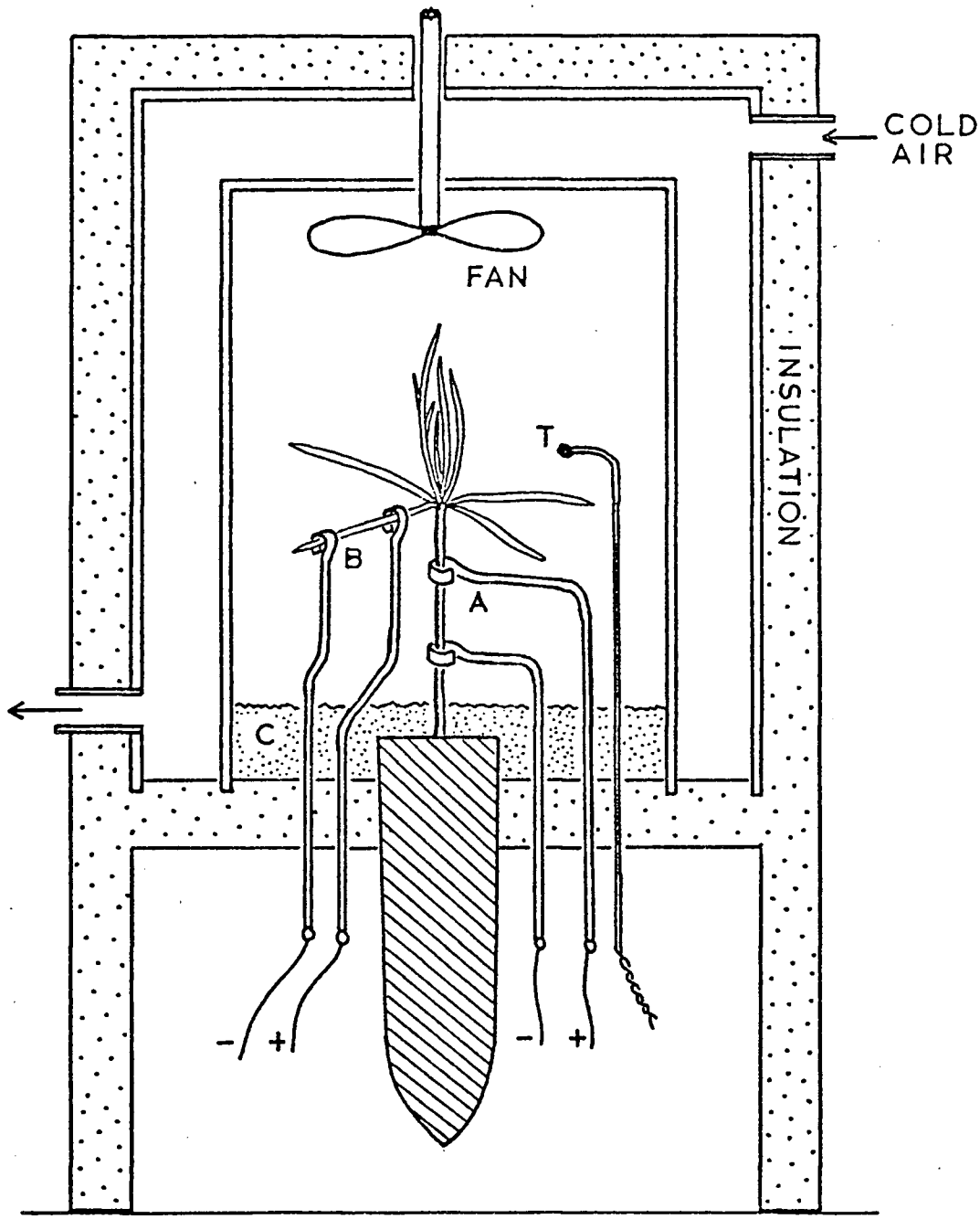

Fig. 3. Freezing chamber for measuring hardiness and rate of ice formation in aerial parts of very young seedlings (Experiment 2). Electrodes partially encircled the stem (A) and a cotyledon (B) (or new needle). Tissue was held in non-injurious contact by a small amount of foam rubber within the electrode loop and a film of electroconductive paste between tissue and metal. Thermocouple (T) recorded ambient temperature. Layer of vermiculite (C) insulated the root system against freezing. The freezing chamber actually contained 8 plants, among which a total of 4 electrode pairs could be distributed.

cotyledons or needles of four plants during each freezing test, a film of electroconductive paste (Burton Parsons Co. "EKG SOL") ensuring good electrical contact between metal and tissue (Fig. 3). Each circuit contained a 3 v dry cell, and a strip chart recorder sensitive to a 10-microamp range of current. Six runs were made at each sampling date, each succeeding run being cooled to a lower temperature so that range from completely safe to completely lethal was obtained. Each run used eight plants of which four were electrically monitored. The more developed seedling type was tested only after the 4-week treatment period.

The results are shown in Figure 4. Prior to elongation of the epicotyl, seedlings were not capable of developing any tolerance of freezing. Measurements of current showed that when ice formed, germinants were invariably killed in spite of short-day, low temperature treatment for as long as 9 weeks. After a similar period, seedlings with 1 to 2 cm of new shoot tolerated freezing, which took place at a more gradual rate. During the course of chilling treatment, however, germinants acquired a greater capacity for supercooling (Fig. 4) and could thus avoid light frosts of short duration. Neither the permanance of this condition, nor the extent to which it might develop outdoors was investigated.

Summary of Growth, Development and Temperature Relationships

The observations of Experiments 1 and 2 for coastal Douglas-fir are summarized in Figure 5. According to this representation, the

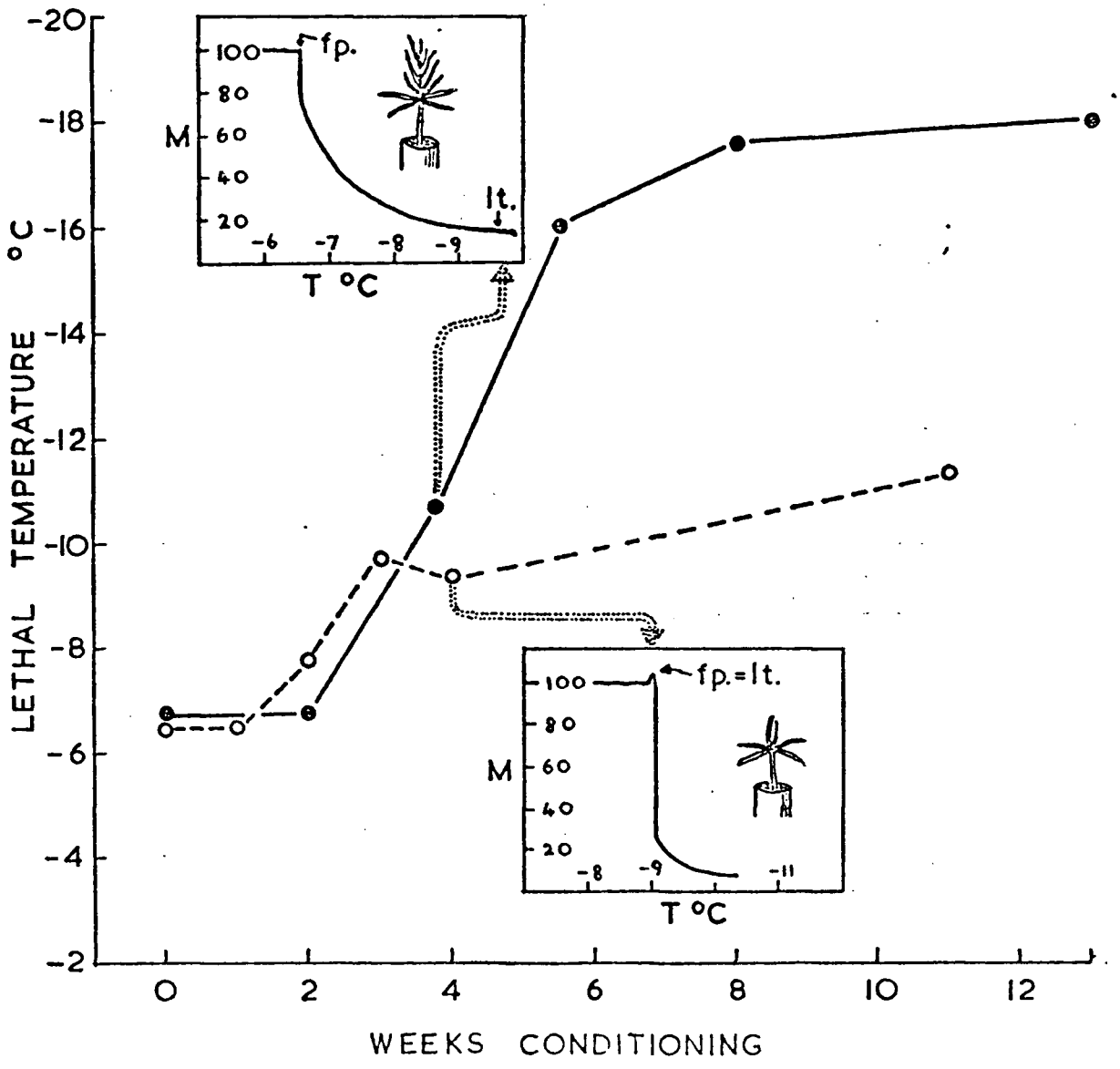

Fig. 4. The cold-acclimation and freezing pattern of 1-week (--0--) and 3-week-old (—0—) seedlings (Experiment 2). The lethal temperature (lt.) is that at which 50% visible injury occurred after freezing. Each point is the mean of 10 to 40 seedlings and the upward trends are highly significant. Inset diagrams represent the relative mobility of liquid water (M) in two seedling types, as a function of temperature during uniform cooling at 7°C/hr. M is the ratio of electrical conductance at temperature T to that at 0°C, multiplied by V_t/V_o, the ratio of viscosities of liquid water at the two temperatures (14). fp is the freezing point after initial supercooling.

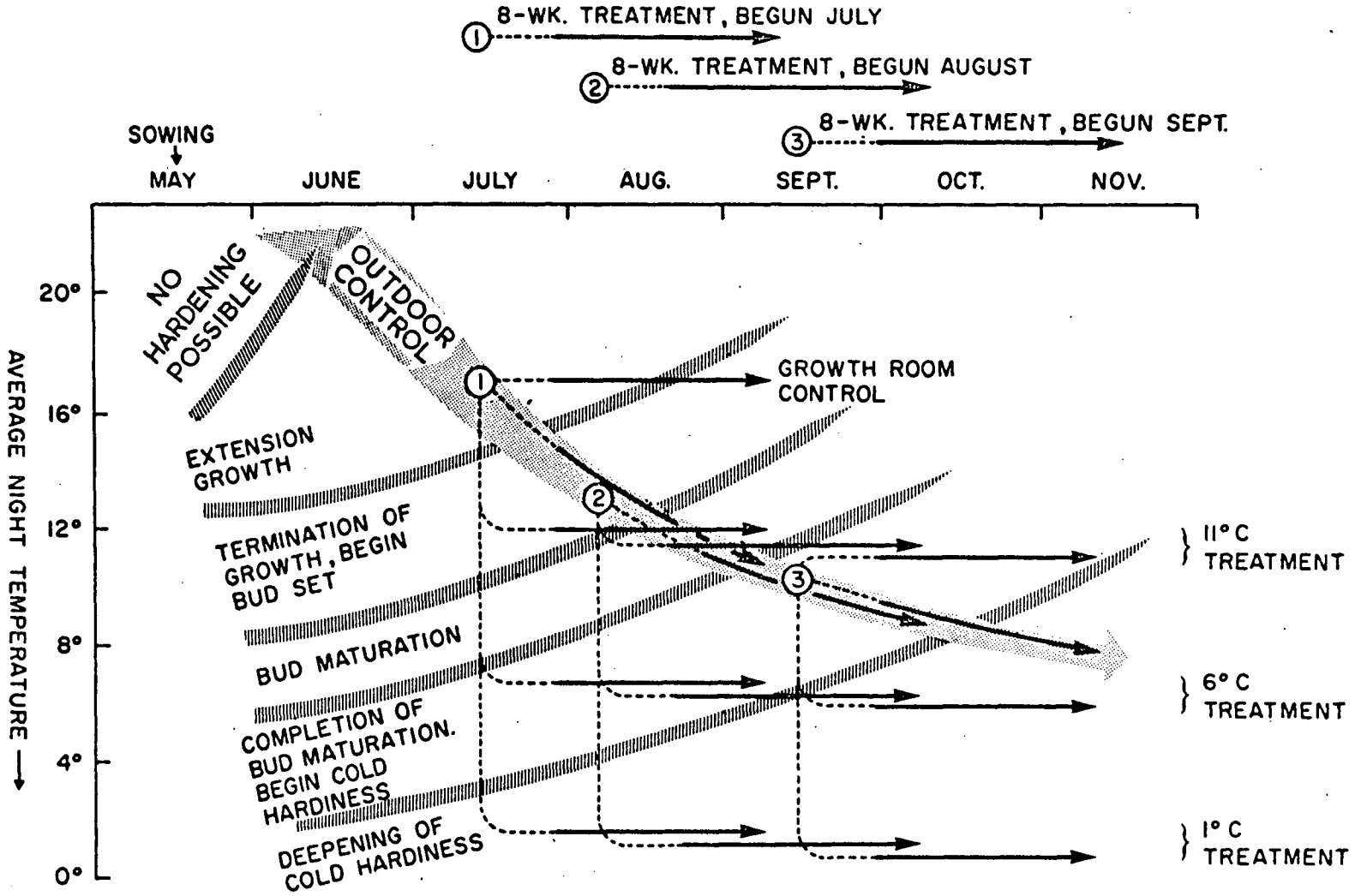

Fig. 5. Schematic relationship of seedling development and hardiness to timing, duration and severity of cold treatments (Experiments 1 and 2). Circled numbers indicate the succession of timing treatments in their initial developmental positions. Arrowheads indicate the growth status at the end of their respective temperature treatments or under outdoor conditions in Victoria, B.C. The dotted portion of the treatment lines represents the 2-week period during which no change in hardiness could be detected (Fig. 4).

final condition of the plant depends principally upon the amount of time spent within the successively lower temperature ranges for extension growth, maturation (bud set, lignification), and cold acclimation. Thus, for example, seedlings exposed to 1°C in July spent no time within the temperature range for completing height growth or maturation, but instead developed hardiness while still small, pale green and succulent in appearance. The 11°C treatment, on the other hand, allowed maturation to proceed but the plants did not enter the cold-acclimation range. September treatments began with fairly mature plants, which under 6°C attained a hardiness equivalent to that induced by the 1°C treatment begun in July; 11°C at this time caused some loss of hardiness by mid-November compared with the outdoor plant. The slope of the arbitrarily positioned growth-stage boundaries allows for a limited endogenous control of development which was observed under constant conditions.

Experiment 3 - Photoperiod and Light Intensity

The effect of photoperiod under the low light intensity of Experiment 1 was to increase hardiness only at the intermediate, 12-hr level. Differences between 8 and 16-hr treatments were not significant, suggesting that a photosynthetic deficiency was limiting acclimation under the normally favourable 8-hr day. This experiment was designed to clarify these relationships.

Twelve plants each of the Mt. Prevost and Prince George provenances were placed under photoperiods of 0, 1, 2, 4, 8, 12, 16, 20 and 24 hr in a cold room at 1±1.5°C for 6 weeks. A light intensity of 800 ft-c was provided in each light-tight compartment by two 40 w fluorescent tubes (one "cool white" and one Sylvania Electric "gro-lux") 20 cm above the plant tops. Air was circulated with fans so that average temperatures around the plants remained uniform irrespective of photoperiod. Single and double layers of shading cloth reduced light intensities to 200 and 50 ft-c respectively in subsections of the 8-hr and 16-hr compartments. Plants were 10 weeks old when treatments began and had not formed terminal buds. Controls, under a 12-hr, 21-15°C day-night regime continued to receive applications of fertilizer. Hardiness was assessed by freezing whole plants, then sampling foliage for relative conductivity determinations (20), and also recording visible injury after a further 4-day period.

Figure 6 shows that 16-hr days were inhibitory to acclimation at each of three light intensities according to two injury criteria after two freezing tests, relative to the 8-hr day. Light intensity became severely limiting below 200 ft-c and increased acclimation at least up to 800 ft-c. The greater acclimating effect of 8-hr days was exhibited under the red-emitting fluorescent tubes of this experiment even at light intensities half those of the non-effective 8-hr incandescent treatments of Experiment 1. This may be associated

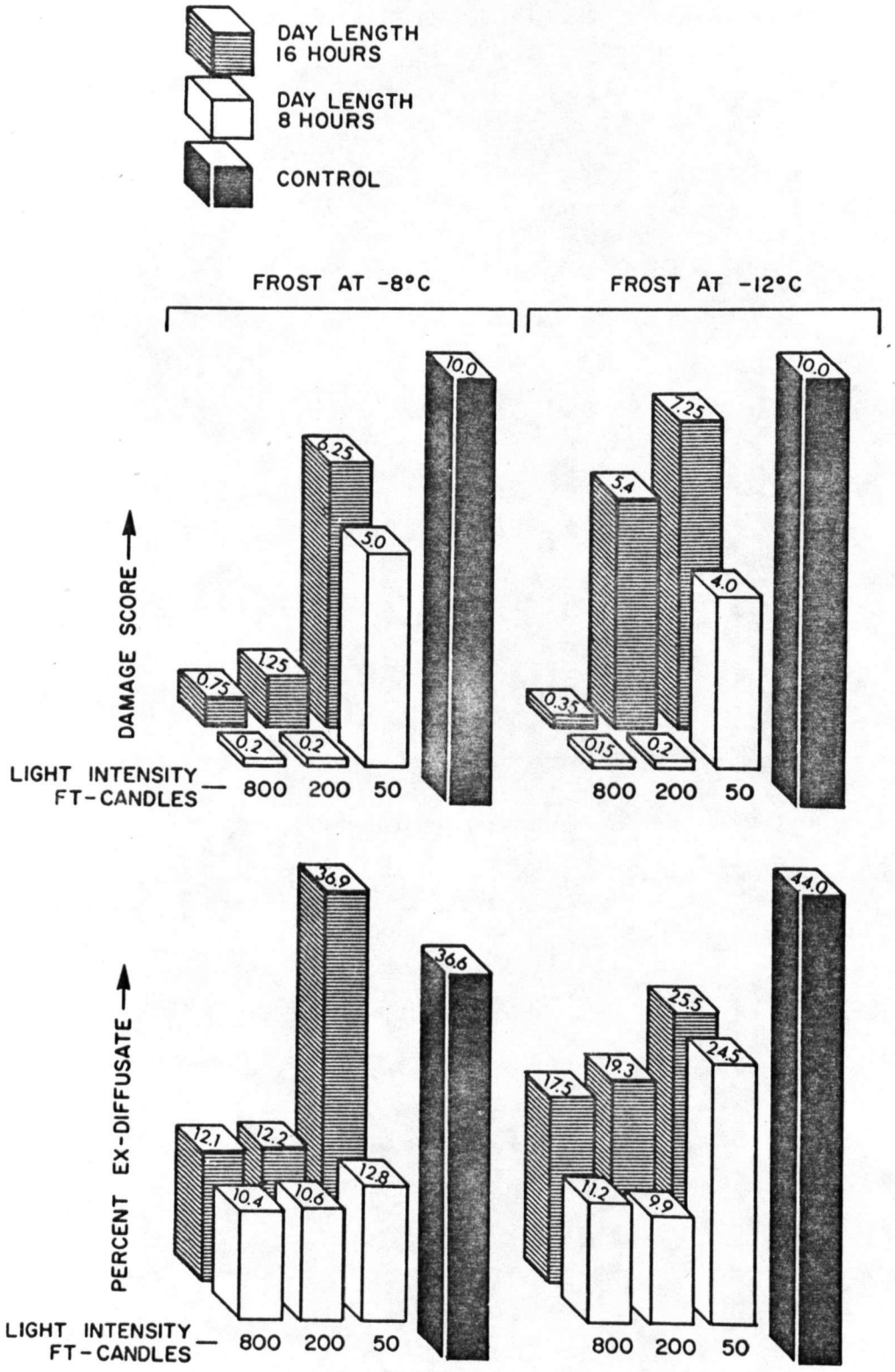

Fig. 6. Effect of light intensity and photoperiod on hardiness (Experiment 3). Controls remained in an environment favourable to growth, while all others experienced 1°C for 6 weeks. Different plants were used in each of the two freezing tests. Numbers on columns indicate average visual score (out of 10) or conductivity percent. Four plants from each treatment were sampled for conductivity, of which two were subsequently assessed visually.

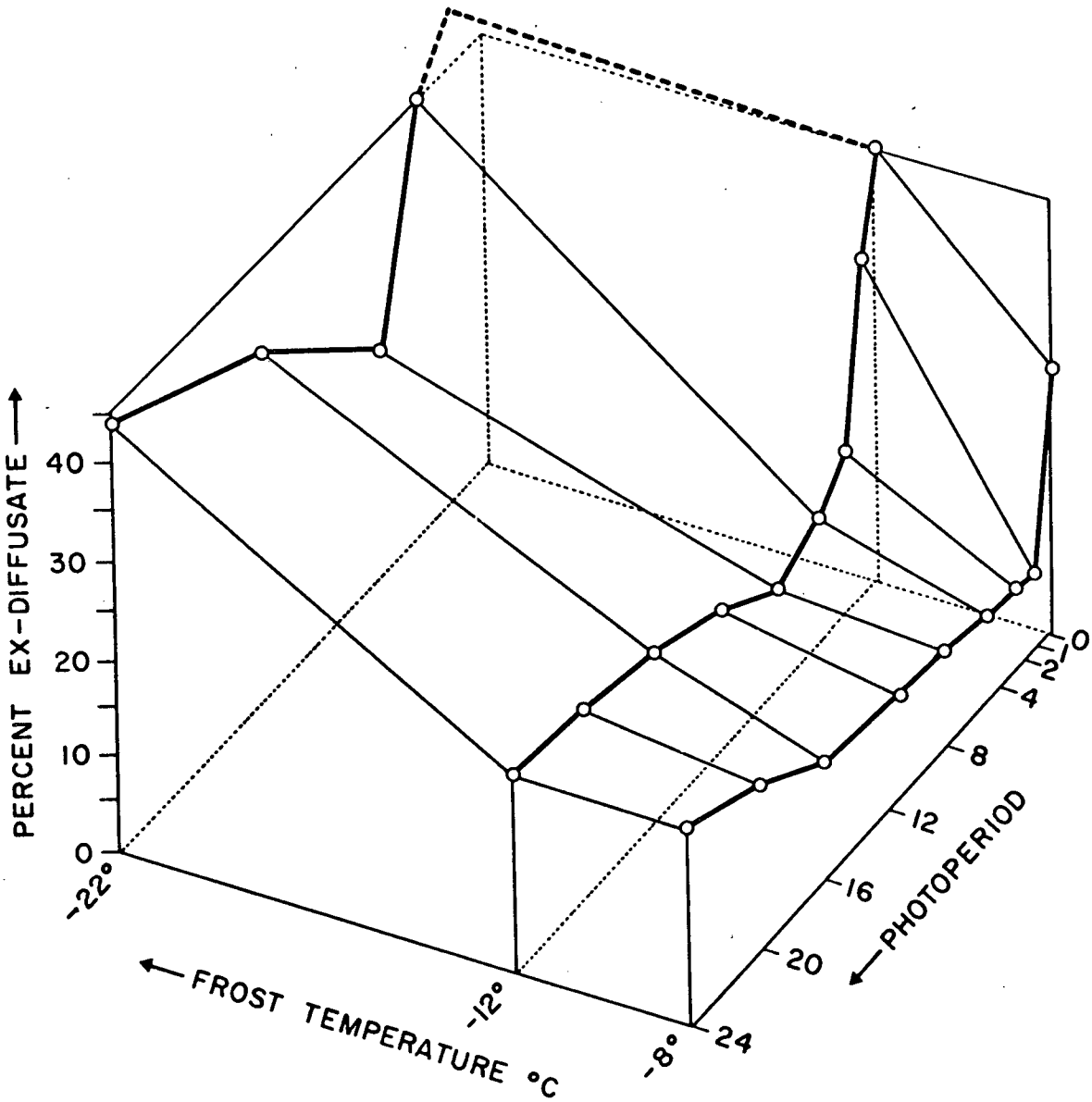

Fig. 7. Freezing injury to foliage after conditioning at 1°C under a range of photoperiods (Experiment 3). Each point is the mean of observations on four plants. The increased hardiness at intermediate daylengths is highly significant.

with the fluorescent emission having (1) a greater proportion of photosynthetically useful light or (2) a greater proportion of red to far-red light. A comparison of the whole range of bright photoperiods for hardiness induction is shown in Figure 7. The trough corresponding to minimum injury under 8-hr photoperiods is highly significant. This optimum was also exhibited by the interior provenance of northerly latitude.

Experiment 4 - Red and Far-Red Night-Interruptions

The aim was to demonstrate the mediation of the phytochrome system in the photoperiod response of growth and hardiness studied earlier. This has been postulated by various workers (e.g., 5, 24), and recently shown by experiment in the broadleaved species Cornus stolonifera Michx. (25). A unique effect of red light (650 nm) which can be reversed by far-red light (730 nm) consititues strong evidence for the participation of this pigment system in a particular phenological process. In the present experiment, as in work on flower initiation (17), the long inductive dark period was briefly interrupted by red (R) and far-red light (FR).

Five treatments were applied by the apparatus illustrated in Figure 8. This consisted of five light-proof compartments each with interior incandescent lighting at approximately 800 ft-c. Four 500 w Sylvania Electric "cool beam" reflector flood lamps mounted externally under a light-proof cover and above a system of colour filter windows, provided directional beams of R and FR

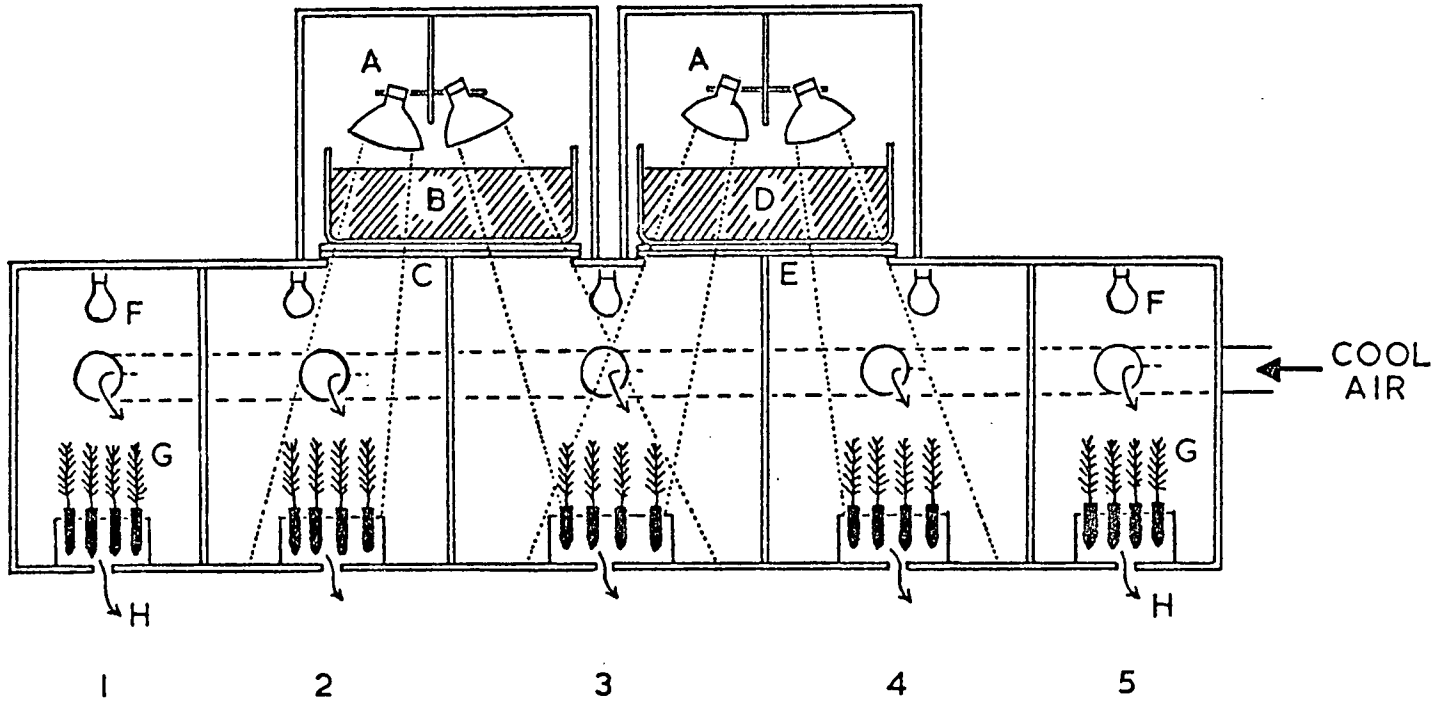

Fig. 8. Apparatus for night-interruption treatments during hardening (Experiment 4). Plants (G) in cooled chambers received long or short days from incandescent lamps (F). 500 w flood lamps (A), with reflectors transparent to infra-red, provided a 15-min burst of red or far-red light. B and C respectively are the liquid and solid filter components for red; D and E, for far-red. Numbers beneath designate the treatment combinations (see text). Air exit vents (H) were light-proof.

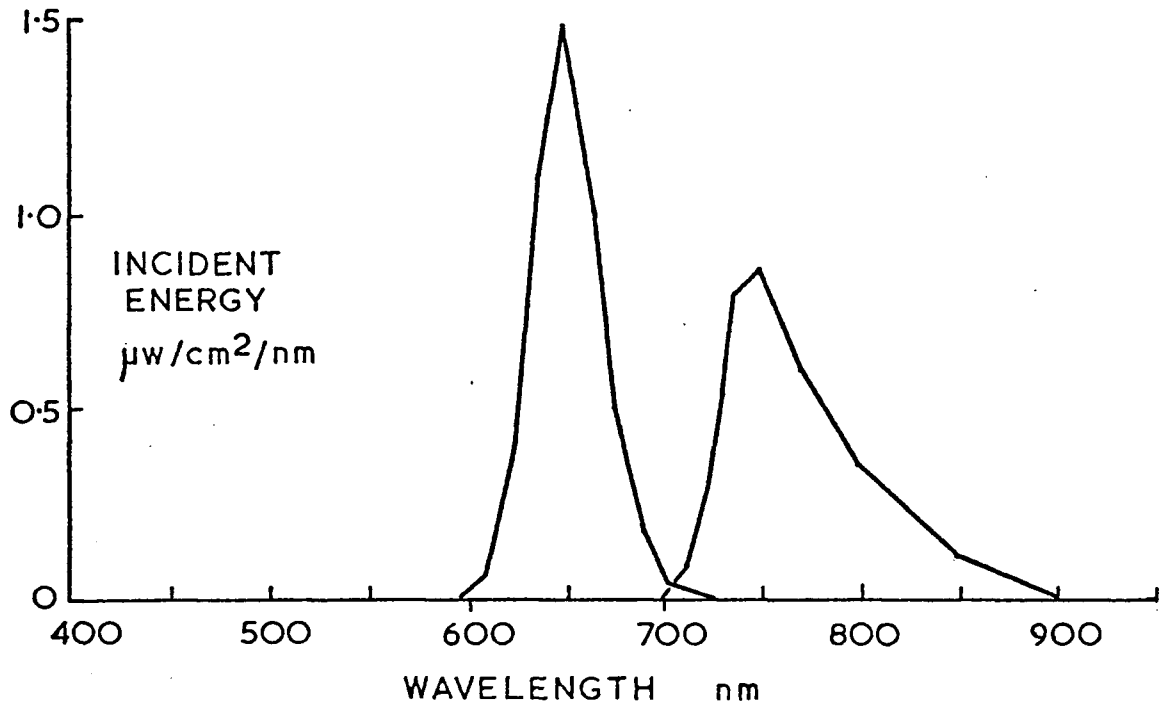

Fig. 9. The spectral distribution of energy from red and far-red sources (Experiment 4). The integral energy (= "intensity") in each case is about 60 μw.

light to the plants. The colour filters consisted of red or far-red Carolina Monochromatic Light Filters (Carolina Biological Supply Co.) in combination with additional components of other CBS Filters, placed beneath a "plexiglass" tank containing infra-red absorbing solutions, in accordance with the system developed by Poff and Norris (15). The treatments were given on a 24-hr cycle for 9 weeks. Treatments 1 to 4 consisted of an 8-hr light period, and a 16-hr dark period which was either (1) continuous, (2) interrupted in the middle by 15 min of R light, (3) interrupted by 15 min of R followed by 15 min of FR, or (4) interrupted by 15 min of FR. Treatment 5 was a 16-hr light period with an 8-hr continuous dark period. The total energy of R and FR bursts, measured as the area beneath intensity/wavelength curves (Fig. 9), was equalized by adjusting the distance of the lamps. The spectrum of radiation intensities was measured at plant height with an ISCO model SRR spectroradiometer (Instrument Specialities Co., Lincoln, Nebraska) and remote probe. The long-day treatment received its additional 8 hours of light from a low intensity (25 w) incandescent source so as not to introduce significant photosynthetic or air temperature differences. Flood lamps were switched on by an automatic timing system for the 15-min period every night. Each compartment was cooled by air conditioners under thermostatic control.

The experiment was repeated three times under different conditions in an effort to obtain clear-cut results. Observations

of bud development and measurements of height growth were made before and after treatments in each case. Initially, groups of 20 3.5-month-old plants, nearing the end of extension growth and bearing incipient buds in some cases, were given the five treatments. R and FR radiation intensities were 50 and 60 $\mu w/cm^2$ respectively, with a 10% variation between chambers and across a beam. Day and night temperatures were respectively 21±4 and 10±4°C, the variation cycling with a 1-hr period. In the second trial, intensities were increased, and the proportion of FR was increased further to compensate for its greater reflectance from the foliage. R and FR had intensities of 570 and 3500 $\mu w/cm^2$ respectively in this case. Three-month-old plants of the Chilliwack provenance were also treated. In the third trial, seedlings at the stage of rapid extension growth (2 months) were treated so that effects on growth and development might be more obvious. The high intensities, which had produced no significantly clearer response, were reduced to almost half their original level at 30 $\mu w/cm^2$ for both wavelengths. The temperature control was improved to eliminate small differences between chambers, and the average night temperature was raised to 16±1°C so that acclimation could not be attributed to cool temperatures. In all cases, at the end of the treatment period needle samples were taken from 10 to 12 plants to determine hardiness by freezing at 3°C temperature intervals and visually assessing injury as described.

Results of moderate and high intensity interruptions of the long inductive dark period are shown in Figure 10. Actively growing 3.5-month-old seedlings under short days had ceased height growth and set buds after 9 weeks, and were about 4°C hardier. Red light interruptions caused a significant decrease in both maturation and hardiness (≈1.5°C) in accordance with the hypothesis that phytochrome is involved. Far-red interruptions alone had no effect, but when applied immediately after the red caused a further, and significant, extension of the red light effect — that is, more growth and lower hardiness. No reversal of the red light effect was demonstrated.

Seedlings beginning treatment in October (Trial 2) were equally tall and dormant after 9 weeks whatever the treatment. In the case of the coastal provenance this was expected because the seedlings were older, with buds set, when treatments began. The high elevation Chilliwack provenance probably completes seasonal growth in response to longer photoperiods because frost occurs earlier in its native site, and presumably it had received this stimulus outdoors before treatments started. However, in both cases, the effect of night-interruptions on cold acclimation was similar to that already described. Reversal by far-red was not demonstrated even at a three-times greater "absorbed" energy level than preceding red ("absorbed" denotes incident minus reflected radiation).

Interruption treatments in the third trial produced plants which were not significantly less hardy nor faster growing than

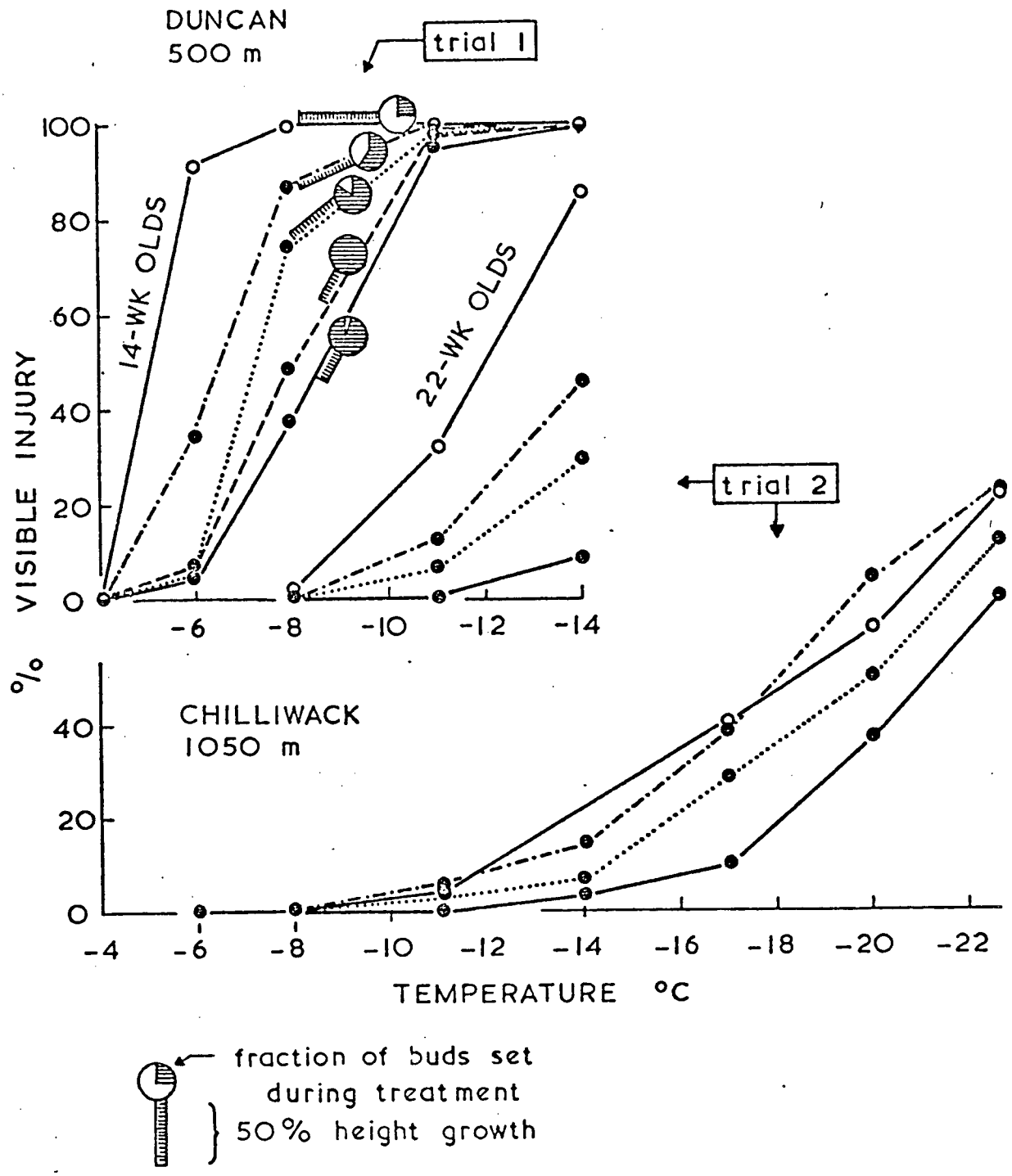

Fig. 10. Effect of night interruptions on hardiness of Duncan (Mt. Prevost) and Chilliwack provenances (Experiment 4). ● short day, ○ long day, —— uninterrupted night, ... night-interruption by 15 min red light, ---- by 15 min far-red light, or -.-.-. by 15 min R followed by 15 min FR. Each curve represents eight plants, each point on a curve is the mean of 10-needle samples taken from the eight plants. The effect of R light in reducing hardiness, and of FR in further reducing hardiness were significant, as were the differences in development in Trial 1.

plants receiving the short-day (uninterrupted night) treatment, presumably because energy levels were below the threshold for response (data are not shown). The differences in hardiness and growth between long- and short-day treatments at the higher average temperature of this trial, however, were greater than in the two earlier trials: 116% height growth, no buds, versus 25% growth, all buds set, for long and short days respectively, and a hardiness difference of 5°C.

Experiment 5 - Sequential Acclimation Treatments

Three treatments, possibly responsible for different stages of acclimation, were applied in various sequences to groups of twelve 3.5-month or 5-month-old seedlings. Treatments were: (0) a greenhouse control with night temperature at 15.5±2°C and day temperatures in the range 17 to 25°C, provided with supplementary incandescent and fluorescent light (800 ft-c) to extend the photoperiod to 16 hr; (1) short days (8 hr, 800 ft-c mixed artificial light), with day temperature 16±0.5°C and night temperature 14±1.5°C; (2) short days as in (1) but with 225±25 ft-c, and a constant chilling temperature of 1.5±0.5°C (2°C with lights on); (3) a short-day chilling treatment as in (2), but with a 6-hr frost at -7±1°C in the middle of the dark period. An additional twelve plants in the third acclimation treatment were kept in continuous darkness (treatment 3D). Temperatures during the nightly frost descended at approximately 10°C/hr and caused ice to form in the leaves.

Plants from the relatively high elevation Mt. Benson provenance were the principal subjects. Treatments began on July 6, 1971, 5 months after germination, at which time height growth had ceased (at about 15 cm) and small terminal buds were set. The total acclimation period of 18 weeks was subdivided into three consecutive intervals of 5, 8 and 5 weeks and the following sequences of treatments defined respectively: 0-0-0, 1-1-1, 2-2-2, 1-2-3, 1-2-3D and 2-2-3. Thus, for example, the 1-2-3 designation means that plants were given 5 weeks of treatment 1 (short days), followed by 8 weeks of treatment 2 (SD + chilling), then 5 weeks under treatment 3 (SD + chilling + night frost). For two additional sequences, 1-2 and 1-3, the acclimation period was considered as being in two parts: 8 and 10 weeks. Excised-needle samples from all plants of the Mt. Benson provenance were measured for hardiness by freezing tests at the end of the 5th, 13th and 18th weeks, and a lethal temperature was estimated for each plant. An additional sample of 5 needles was removed from each plant after the 18th week. These were weighed fresh ($\pm$0.05 mg) and after oven-drying at 105°C for 24 hr ($\pm$0.02 mg) to determine water content.

Smaller, 3.5-month-old Mt. Prevost seedlings (averaging 8 cm in height and with buds not set) were placed under some of these treatment sequences, totalling only 12.5 weeks duration and beginning 21 July. In this case the intervals were 5, 5 and 2.5 weeks for sequences 0-0-0, 1-1-1, 2-2-2, and 2-2-3; and 7.5 and 5

weeks for the 1-2 treatment sequence. Hardiness of these plants was measured only at the end of the 12.5-week period. All plants were returned to the control environment (treatment 0) for a 2-month period after final hardiness sampling, to assess the extent to which treatments had induced or broken rest.

The effect of sequences in which short-day, chilling, and night frost treatments were applied is shown in Figure 11. Plants under warm long days (0-0-0) did not flush, thus indicating that all plants had entered rest prior to treatment. Growth tests afterwards showed that all chilling treatments had broken rest.

Chilling temperatures in conjunction with short days (2-2-2) caused most rapid acclimation, and this developed mainly within the first 5 weeks. Prior exposure to warm short days (1-2) did not increase the effectiveness of the chilling treatment in spite of the fact that the short-day treatment alone caused a change in appearance (darker, rigid and more spreading leaves, woodier stem) and a moderate increase in hardiness of 5°C. Night frost treatments caused considerable deepening of hardiness if plants had first received a period of chilling, and if light continued to be supplied (1-2-3, 2-2-3). Frosts applied directly after the warm short-day treatment (1-3) caused death of most individuals, although resulting in further acclimation of those which survived. This acclimation, however, was not significantly greater than that of plants only chilled during the same period (1-2). Stage 3 treatment in

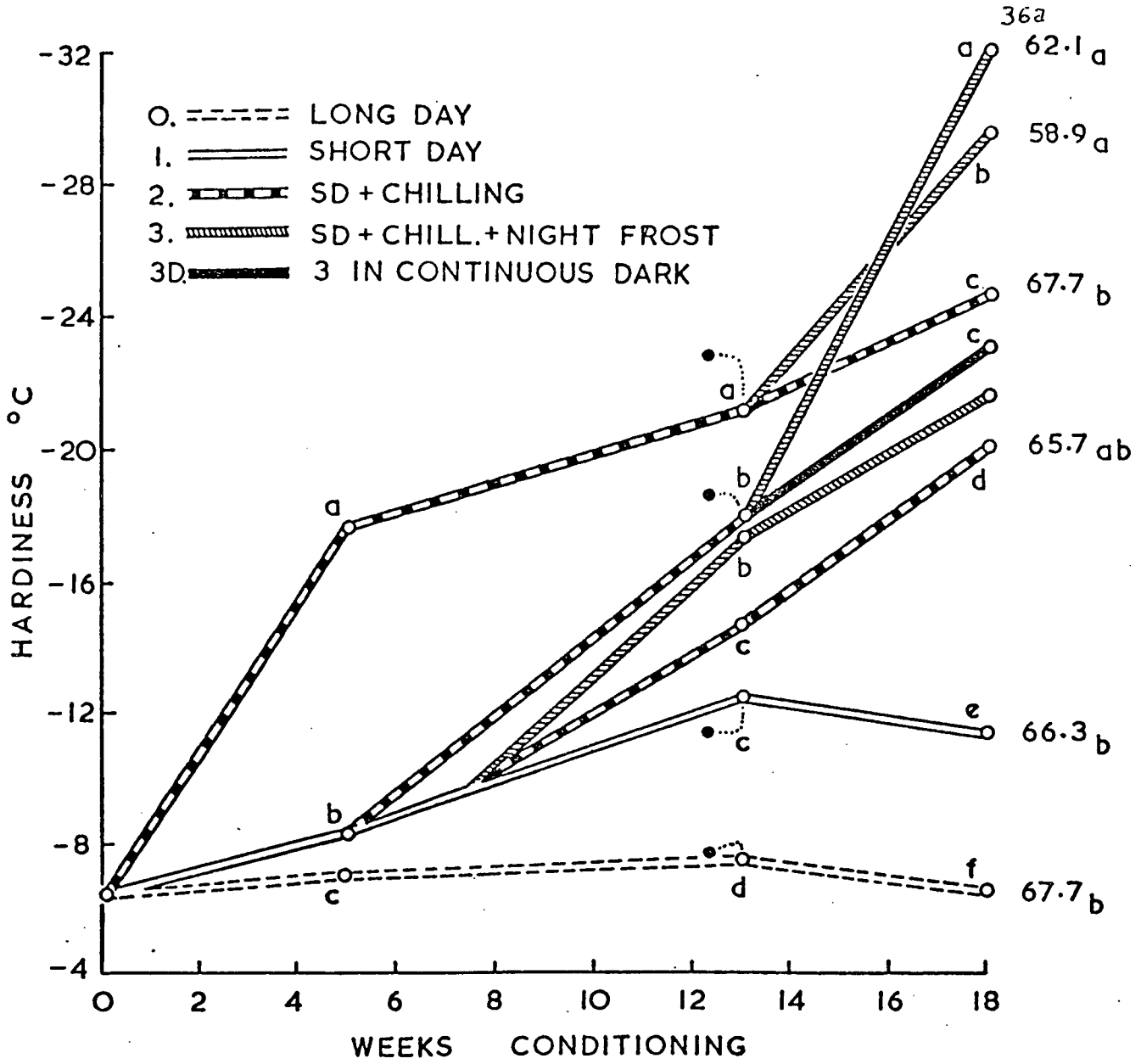

Fig. 11. Effect of treatment sequence on hardiness of the Mt. Benson provenance (Experiment 5). Number of plants in each treatment combination at 18 weeks was 12, except in the 1-3 combination (which was omitted from the analysis at this stage due to low survival). Points bearing the same letter do not differ significantly at the 5% probability level (this analysis was conducted for each treatment duration independently). Numbers denote foliage water content (fresh weight basis, and where sufficient foliage remained for sampling) at the end of treatment, and common subscript letters indicate the lack of significant difference between them. Additional points, ●, represent hardiness of Mt. Prevost plants at 13 weeks, after corresponding treatments.

continuous darkness (1-2-3D) increased hardiness to a much lesser extent and caused the foliage to abscise. Maximum hardiness was attained under the conditions most resembling those in nature (1-2-3). This was significantly greater than hardiness under 2-2-3 and the plants were acclimating at a faster average rate when the experiment ended. Water content, on a fresh or dry weight basis, of foliage subjected to these two freezing treatments was significantly lower, whereas among most other treatments water contents were similar (Fig. 11). Yet if these leaves were rehydrated to "normal" water levels by a 14-hr immersion in cold water, their hardiness remained unchanged (data not shown).

The younger and relatively low elevation Mt. Prevost provenance responded in a similar way (Fig. 11) with the exception that night frosts inflicted gradually increasing injury, so that the effect of this factor on hardiness could not be evaluated.

DISCUSSION

The relative effects of temperature, photoperiod and light intensity on hardiness reported above are in close agreement with those found in older seedlings of this species after cessation of growth (5, 6). Maximum acclimation under a single treatment occurred at low positive temperatures and short (8-hr) days. Low light intensities had a longer optimum photoperiod (12-hr), and very short days (less than 6 hr) reduced hardiness whatever the light intensity — all in accordance with the accepted view that

there is both photoperiodic and photosynthetic control of acclimation. Upper and lower threshold levels of light intensity are also in agreement with reported values (6, 26). Long-day inhibition was important only at higher temperatures.

The inability of germinants to acquire freezing tolerance (Fig. 4) does not appear to have been previously noted. The acquisition of freezing avoidance in them is most probably an artifact of reduced water flux through the plant and slight dehydration, which occurred in the cold room — water content and supercooling are frequently related. The true acclimation at slightly later stages of development (Fig. 1) clarifies several points. (a) Since both non-dormant (July) and dormant (September) plants acclimated at low temperature, then the process does not depend on entry into rest. This qualitative independence has been demonstrated (10) in Viburnum plicatum Miq. (b) The short-day treatment was equally effective on both actively growing plants, and those with buds set and in rest. Moreover this treatment, while markedly affecting hardiness, had no effect on development under low light intensity and cool conditions. Therefore, the short day does not increase hardiness by inducing rest or promoting bud development and lignification, but does so independently. (c) The significantly greater hardiness induced at later stages of seasonal development (Fig. 4) appears to be endogenously superimposed upon environmental effects. That it

is not a result of exposure to shortening days outdoors (before treatments began) is shown by the failure of warm, short-day treatment for 5 or 13 weeks to increase the acclimation during subsequent chilling (Fig. 11). An endogenous seasonal rhythm of hardiness, (i.e., under constant conditions) has been demonstrated in Pinus cembra L. (19) and was also indicated by observations on red osier dogwood (7) and Haralson apple (8). Such rhythms can be ones of inducibility rather than hardiness per se. (d) The apparent opposition between development and cold-acclimation (Fig. 2) is most simply viewed as a consequence of differing temperature optima rather than a production of hardiness inhibitors during growth. Figure 5 incorporates only the endogenous rhythm and temperature effects because no photoperiodic control of development was observed in Experiment 1, although this became significant at higher light intensities and temperatures (Fig. 10), as found by others (11, 17, 23).

The effect of night interruptions by red light in bringing about a significant reduction in hardiness suggests, but does not prove, a phytochrome control because the red light effect was not shown to be unique among visible wavelengths, nor reversed by far-red. The total energy supplied was two or three orders of magnitude above the threshold for the flowering response in other species. In the second trial the FR intensity was sufficiently high (3500 $\mu w/cm^2$) to compensate for its two times greater reflectance from

the leaves (measured with the ISCO), the lower absorption by P_r as opposed to P_{fr} (x 1.5), and the lower quantum efficiency of $P_r \longrightarrow P_{fr}$ conversion (x 1.5) compared with the reverse process (17). It therefore could not simply have been energetically insufficient to reverse the effect of red. In fact the total FR energy supplied in Trial 2 was close to the level of the "high energy reaction" (HER) in which FR or blue light can produce effects similar to lower intensities of red (17). However, the similar FR enhancement of hardiness at 60 uw levels in Trial 1, and typical reversal effects on hardiness shown at much higher energies in red osier dogwood (25). both suggest that the HER was not a complicating factor here. Prolonged treatment of plants with FR of low intensity sometimes causes effects identical with those of red (17) and Dinus' data for Douglas-fir seedlings provide such an example (4) — albeit at 30-min exposures rather than the 15-min exposures used in the present study. So it appears likely that the present FR enhancement might be a further case of this long-exposure phenomenon, which is, however, still explainable in terms of the phytochrome system (17).

The greatest acclimation under a sequence of treatments (Fig. 11) occurred when these corresponded roughly with the three stages summarized by Weiser (24). When only short-day and chilling treatments are considered, there was more or less complete independence: chilling for 11 weeks brought hardiness to a certain

level regardless of preceding warm short days with their accompanying morphological changes; short days induced a distinct, constant but lower level of hardiness which was not additive to subsequent chilling effects — at least in the balance of the 4.5-month treatment period. Both treatments, however, appeared to be necessary for acclimation at freezing temperatures because hardiness under 1-2-3 exceeded both 2-2-3 and survivors of 1-3 (which were no hardier than plants given 1-2). It is thought that the benefit of warm short days was connected with their obvious effect on morphology, which seemed to confer better long-term protection against repeated freezing. Whether the further acclimation of periodically frozen tissue constitutes a third "stage" in the physiological or biochemical sense, as opposed to a closer approach to the temperature optimum for a single cold-induced process, was not definitely established. Three points favour the hypothesis of a third stage: (a) the dual short day and chilling prerequisites, (b) the somewhat faster response, and (c) the concurrent dehydration as a possible inductive factor distinct from temperature (12). Alden's data (1) also indicate that the sub-freezing response of cut Douglas-fir twigs in the dark was associated with dehydration. A separate and relatively short-term physiological response to sub-zero temperatures, which requires light, has also been indicated by hardiness and gas exchange measurements in this species (18).

The initial independence and eventual reinforcement of the hardening factors (endogenous, short-day, chilling and freezing) must provide a valuable adaptive flexibility to the plant in nature, particularly at higher altitudes and inland. In this respect there is good agreement with the acclimation process in apple (8). From a practical viewpoint, it is more important that hardiness can be induced by a single treatment at any stage after initial epicotyl growth. Such a "minimal acclimation" might allow overwintering of plants soon after greenhouse germination, thereby permitting a more continuous schedule of sowing and other nursery operations. It would also permit pre-adaption of groups of mature seedlings destined for relatively cold planting sites.

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CHAPTER 3

TRANSLOCATION OF DEHARDENING AND BUD-BREAK PROMOTERS IN CLIMATICALLY "SPLIT" DOUGLAS-FIR

ABSTRACT

Forked Douglas-fir seedlings were exposed to warm (20°C) and cold (2°C) environments simultaneously, by inserting one branch in each environment. All received the same light conditions. Hardiness of foliage after one to five months was measured as the freezing temperature causing 50% visible injury in excised needles.

The chilling stimulus for breaking rest and inducing hardiness was confined to the chilled branch, but the warm branch apparently transmitted a factor which prevented full hardening in the chilled one. A factor moving in the same direction also promoted flushing in branches chilled only at night from December to June (and receiving greenhouse temperatures and natural photoperiods by day). This was not replaceable by a single injection of gibberellic acid. Factors from the expanding shoot caused loss of short-day-induced hardiness in previous year's foliage and stimulated cambial division. Chilling at night prevented the dehardening but did not prevent cambial activity. The dehardening factor was translocated to an opposite branch whereas movement of cambium stimulator was strictly basipetal.

It is suggested that promoter-inhibitor levels controlling dormancy are independently regulated, and that a two-stage

dehardening process might protect against premature loss of hardiness in nature. The simultaneous induction of cold acclimation and dehardening in the same plant provides material for comparing properties of hardy and nonhardy tissue.

INTRODUCTION

Recent studies have demonstrated that, whereas the short day (SD) induction and long day (LD) inhibition of hardiness are translocatable in broadleaved woody plants (6, 7, 8, 10), the cold stimulus for deeper acclimation is not (8). The loss of hardiness in Douglas-fir has been attributed only to rising temperature (4), but in other species it also depends on the state of winter dormancy or "rest" (11) — although rest has little influence on the acquisition of hardiness (18). Questions thus arise as to whether there are other dehardening stimuli in conifers, whether warm temperature effects are translocated, and what might the precise relationships be between environment, shoot growth phenology and hardiness in spring. Such relationships have practical importance in forest nurseries where provenances are being raised away from the environment to which they are specifically adapted. The overall aim of the following investigation was to confirm the suspected (and, at that time, unreported) localized nature of the chilling stimulus, with a view to obtaining nonhardy and highly hardy leaves on the same plant for comparative studies. The actual results, however, provide some insight into the relationships discussed above.

GENERAL METHODS

Two-year-old plants were pruned at the beginning of their second growing season to produce forked tops. When the branch

pairs had attained a length of 5 to 10 cm in a greenhouse and entered rest under natural photoperiods (November), the plants were incorporated into one of three experiments involving exposure of each member of a branch pair to different temperatures under the same light conditions (Fig. 1). In two of the experiments, pots were arranged with respect to the two environments so as to have equal numbers of "split" plants with warm as with chilled root systems and stem. Possible effects of root and stem temperature on hardiness and flushing could then be assessed. Controls, with all parts chilled or all parts warmed, were also included. Foam rubber ensured a non-injurious and convection-free seal between bark and plexiglass at the junction between environments. Air was circulated both internally and externally by fans so that temperature gradients did not develop.

Hardiness was evaluated by a series of freezing tests, on samples of 10 excised needles, to temperatures spaced at 3°C intervals across the expected lethal range. Injury was assessed visually after 7 days and hardiness interpolated as the temperature corresponding to 50% injury as previously described (17). A 1-cm length of branch immediately on each side of the plexiglass was excluded from the sampling operation because of possible effects of heat conduction along the branch tissues. Where both branches received identical treatment, only one branch was

sampled for hardiness. Analysis of variance was performed assuming that all observations were independent (although greater specific sensitivity would have been permitted by treating the "divided plant" observations in pairs). Duncan's Multiple Range test was used to distinguish individual means.

EXPERIMENTS AND RESULTS

The essential features of the three experiments are illustrated in Figure 1. All plants had entered rest prior to beginning treatments.

Experiment 1

In the first experiment, conducted on plants of a southeast Vancouver Island provenance, a chilling treatment was applied to one branch (1.5±0.5°C) while the other was kept warm (21±1°C). Mixed incandescent and fluorescent light (400 ft-c) was supplied during an 8-hr day. Temperatures were 0.5°C higher in the light. After 8 weeks, the chilled branch was about 9°C hardier on average than the warm one on the same plant (curves C and D in Fig. 2). Warm branches had acquired the same moderate degree of hardiness (-17°C) under short days whether or not they had cold partners — showing that the effect of chilling was not translocated. But chilled branches with warm partners were significantly less hardy (by about 5°C) than branches of wholly chilled plants (curve E, Fig. 2), showing that some translocated factor was involved. There were no significant differences in hardiness

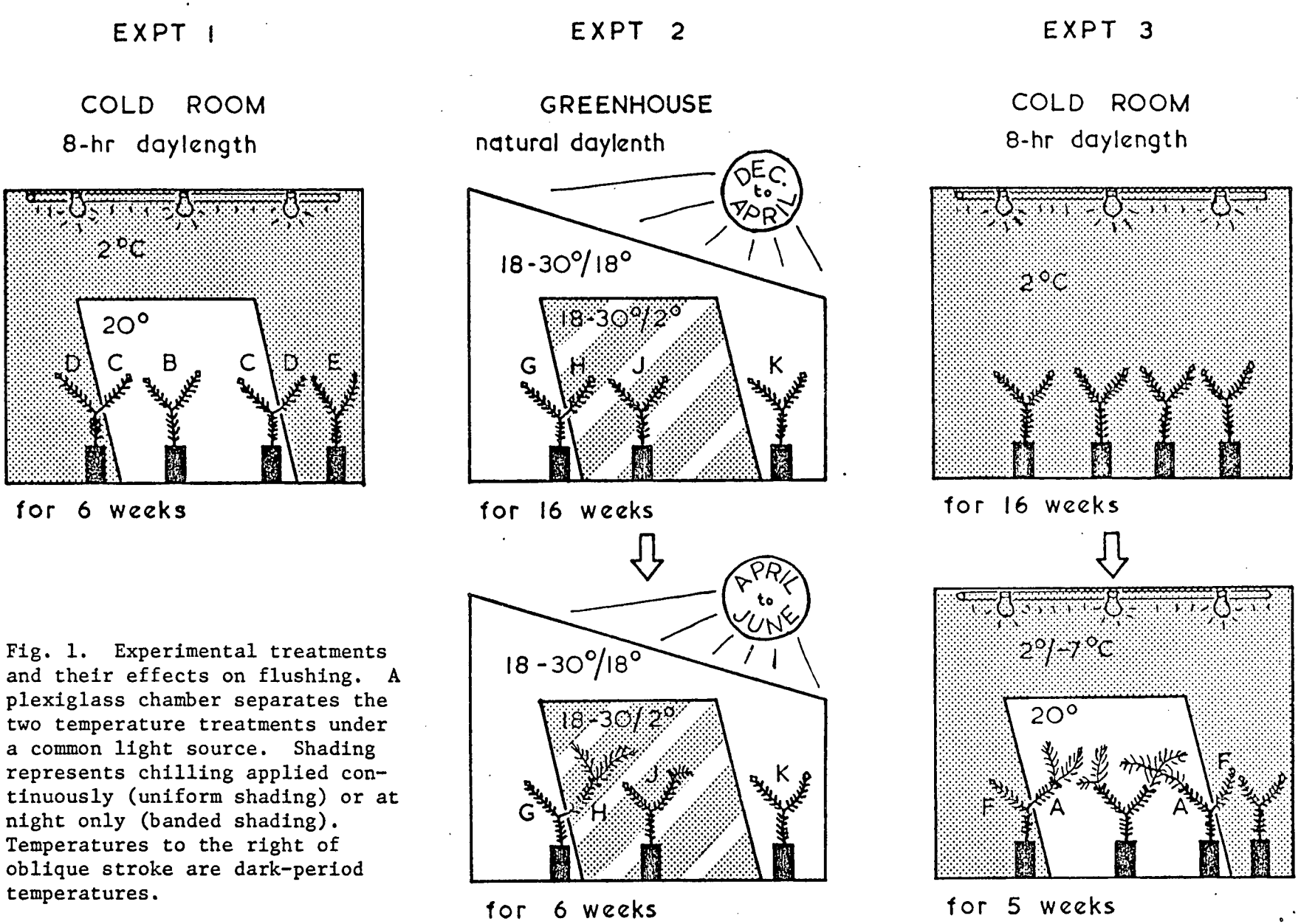

Fig. 1. Experimental treatments and their effects on flushing. A plexiglass chamber separates the two temperature treatments under a common light source. Shading represents chilling applied continuously (uniform shading) or at night only (banded shading). Temperatures to the right of oblique stroke are dark-period temperatures.

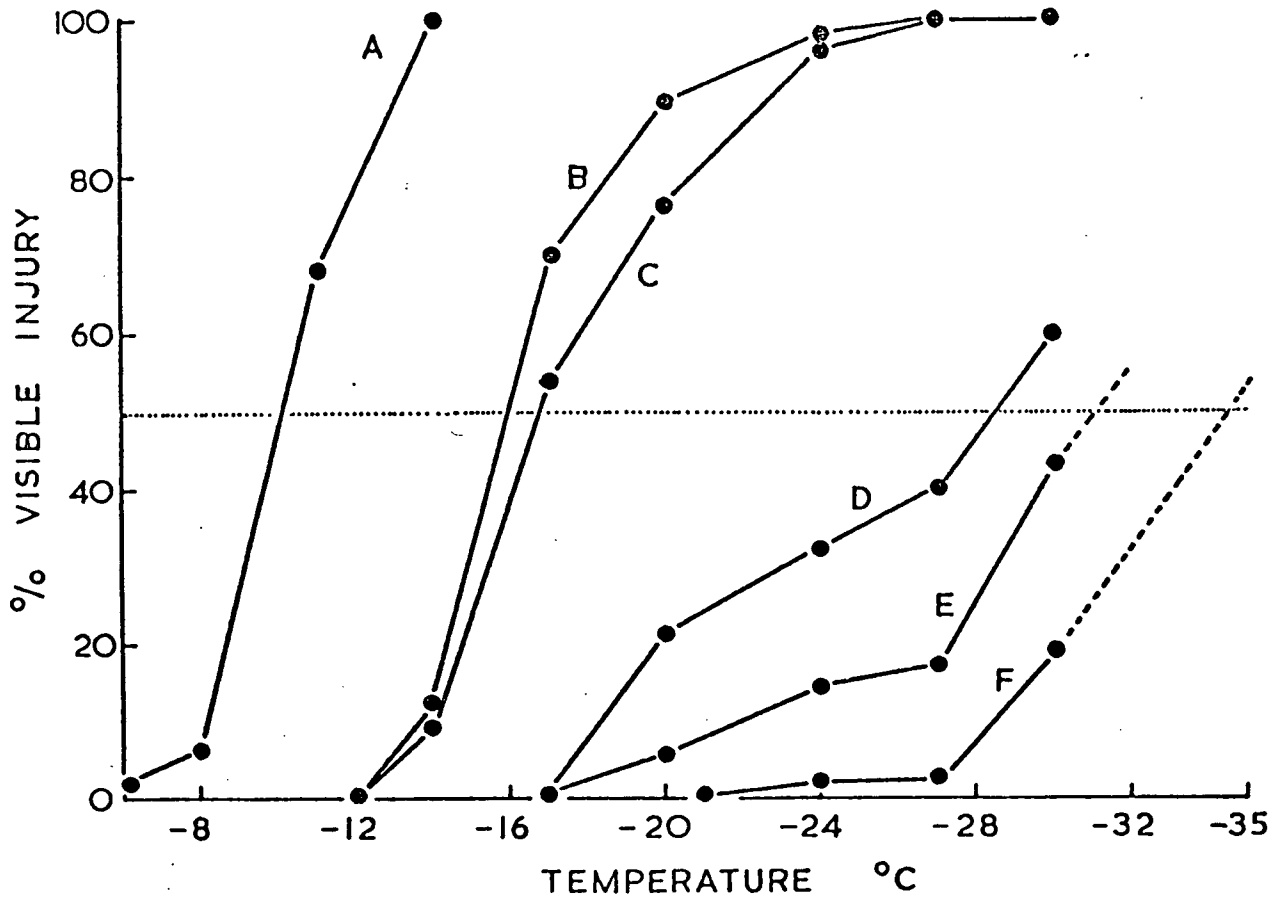

Fig. 2. Hardiness of foliage on climatically split plants. Letters identify treatments shown in Figure 1. Injury curves B and E each represent 10 branches; C and D each represent 20 branches because data for plants having warm roots and chilled roots (Table I) were pooled. A and F are means of 7 branches. Each point on a curve is the mean of 10-needle samples from each branch. Only treatments B and C did not differ significantly at the 5% level.

Table I. Effect of Root and Stem Temperature on the Foliar Hardiness of Warm and Chilled Branches in Experiment 1.

Roots and Stem Chilled			Roots and Stem Warm		
Seedling No.	Hardiness °C		Seedling No.	Hardiness °C	
	Chilled Branch	Warm Branch		Chilled Branch	Warm Branch
1	-32.0	-24.5	11	-22.0	-15.0
2	-19.5	-14.5	12	-28.0	-15.5
3	-26.0	-24.0	13	-23.5	-15.0
4	-31.0	-15.5	14	-22.5	-16.0
5	-21.5	-15.5	15	-26.5	-16.5
6	-27.0	-20.5	16	-28.5	-18.0
7	-32.0	-22.0	17	-22.5	-18.0
8	-19.5	-14.0	18	-31.5	-18.0
9	-25.0	-17.5	19	-29.0	-19.0
10	-31.5	-20.0	20	-29.0	-17.5
Mean	-26.5	-18.8		-26.3	-16.8

associated with the position of the root system and stem with respect to the two environments (Table I).

Experiment 2

Plants of an interior British Columbia provenance (Cranbrook) were exposed to the natural photoperiods of Vancouver, B.C. in a greenhouse during the winter hardening and spring dehardening periods of 1970-71. Chilling was carried out only during a 15-hr period which coincided approximately with the natural night. Under this arrangement the warm branch (and the "all warm" controls) received a night temperature of 16.5±2°C, while the chilled branch (and corresponding controls) experienced 3±2°C during the same period. Air temperatures around all branches during the day depended on the intensity and duration of sunlight, and increased gradually during the period December to June (Fig. 3).

Swelling of buds was observed (April 9) only on those branches which had been chilled and had a warm partner. Relative hardiness of branches under the different treatments at about this time (April 22) was similar to that in Experiment 1 (confirming the translocated "warm branch" effect): warm branches ≈ -17°C, chilled branches with warm partners ≈ -25°C, chilled branches with chilled partners ≈ -30°C (Fig. 3). Hardiness in the foliage of all-warm plants 6 weeks later (June 8) had not significantly changed, but had decreased significantly in warm branches with chilled (and flushing) partners. Moreover, this new low level of hardiness

was the same as that of old foliage on outdoor plants (same provenance) which had flushed at about the same time outdoors. Finally, there was no significant loss of hardiness in the (old) foliage of chilled branches bearing a new flush. By June 8, only one third of the wholly chilled plants had flushed, and these shoots failed to elongate by more than 1 or 2 cm. These relationships are shown in Figure 3, and provide evidence for the following hypotheses. (1) Flushing out is dependent not only on the breakage of rest by chilling, but also upon a translocatable factor(s) produced during warm nights. Only the chilled branch with the warm-night partner received both. (2) A second factor is produced during the expansion of new shoots, which can cause loss of hardiness in older foliage to which it is translocated. This accounts for some of the dehardening of outdoor plants in spring, and the loss of SD-induced hardiness in warm branches with chilled (and flushing) partners. (3) The effect of this second factor is inhibited by low night temperatures, because old foliage on the chilled branch beneath a new flush failed to deharden. Under Hypothesis (1) it was expected that "warm-factor" levels in branches of wholly chilled plants would eventually build up and permit flushing, as in fact occurred to a limited extent (Fig. 3). Injections of 200 µg gibberellic acid (GA 3) in 10 µl ethanol 1 cm below the buds of three trees on June 26, however, failed to bring about any advance in flushing date when compared with controls receiving ethanol only.

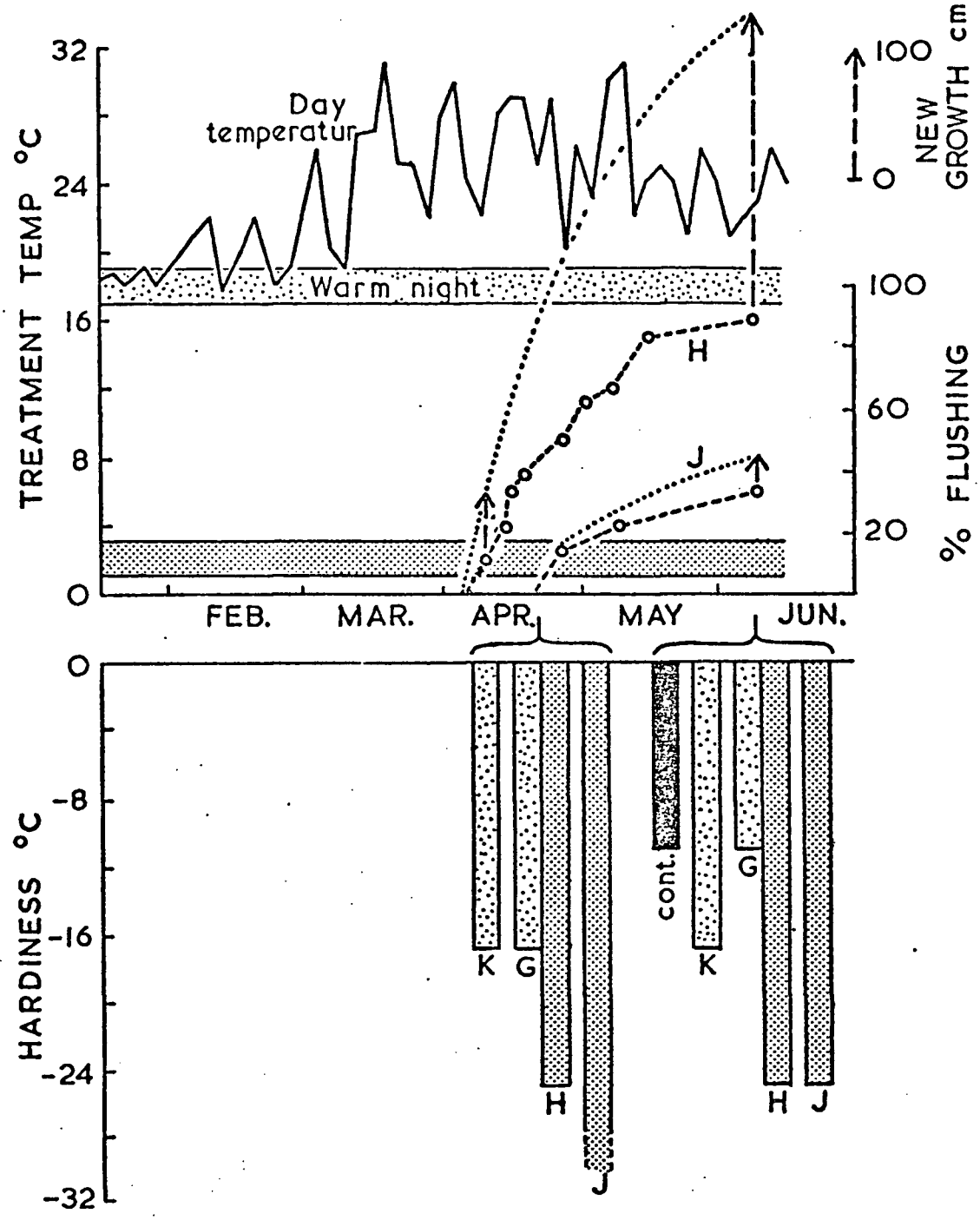

Fig. 3. Hardiness and flushing of an interior provenance under natural photoperiods in a greenhouse (Experiment 2). Night temperature ranges and 3-day averages of daily maxima are plotted in the upper axes, together with percentage of plants flushing (---0--), and the total length of new growth from all buds (vertical arrows). Letters identify treatments in Figure 1. Lower axes show corresponding foliar hardiness with contiguous histogram columns representing warm and chilled foliage on the same plant. Outdoor (flushed) plants were measured in June (cont.). Treatments G and H represent 18 plants; K and J each represent 7 plants. All hardiness differences are significant at the 5% level, as are differences in flushing date and average length of new growth per plant between H and J.

The relationship between dehardening (Hypothesis 2) and cambial activity (which is also stimulated during bud expansion) was examined by sectioning the branches and stem. These sections showed a clear absence of new xylem in the unflushed member compared with new wood in the flushed one. In the upper part of the stem there was cambial activity only on the side directly below the flushed branch, although this xylem segment increased in girth and decreased in radial thickness further down (Fig. 4), presumably due to slow lateral transport and dilution of the stimulating hormone.

Experiment 3

The effect of flushing was to cause a loss of hardiness, while the effect of sub-zero temperatures in concurrent experiments (18) was to increase hardiness beyond that obtainable by chilling. These observations prompted the third experiment, with the aim of obtaining greater differences in hardiness between branches of the same plant and providing a test of hypotheses (2) and (3).

Plants (of Vancouver Island origin, as in Experiment 1) were first subjected to a 16-week chilling treatment, under the same conditions as all-cold controls in Experiment 1, to break rest in both branches. They were then placed in the differential temperature apparatus under the same temperature treatments as Experiment 1 (i.e., continuous, uniform warm or chilling temperatures), except that in the chilling treatment a 6-hr frost at $-7 \pm 1°C$

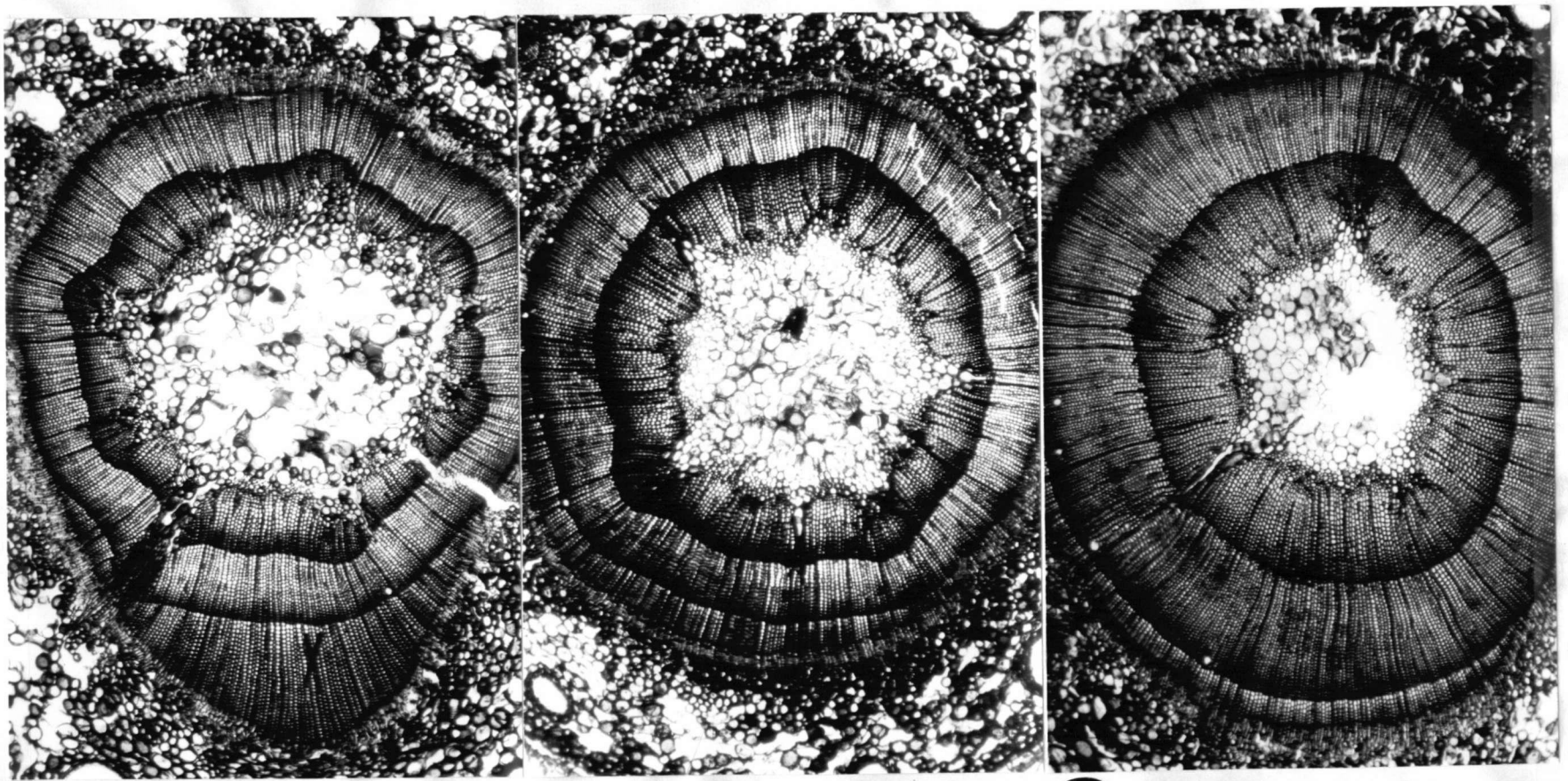

Fig. 4. Stem cross sections of a climatically split seedling bearing a flushing and a dormant branch (x27). New xylen (X) formed directly beneath the flushed branch (1), and was still observable as a one-sided response in both middle (2) and basal (3) sections. Distance between (1) and (3) was approximately 7 cm. Stems were harvested 8 weeks after flushing.

occurred in the middle of each 16-hr dark period. It was established that needles were frozen rather than supercooled during this period.

Flushing began in the warm branches after 3 weeks and was not affected by the temperature of the opposite branch or the roots. Of 20 split plants only the warm branches of three failed to flush in 5 weeks. Two of these were in the "cold-root" group. Hardiness was evaluated only in split-plant branches due to lack of time. Hardiness of old foliage of flushed branches after 5 weeks was slightly lower than that in dehardened outdoor plants or dehardened warm branches (partner flushed) of the preceding experiment (-10°C). Hardiness in chilled-frozen branches was about -35°C (Fig. 2, curves A and F). Thus, the translocatable dehardening factor produced during shoot expansion is, in this case, demonstrated by a comparison of curves A and B in Figure 2. The inhibitory effect of localized chilling over this factor was shown in this case by failure of the cold branch to deharden. The additional hardening effect of freezing temperatures demonstrated in an earlier study (18) was confirmed. A difference in hardiness of approximately 25°C was obtained between branches.

DISCUSSION

The localized nature of chilling in inducing hardiness, as shown in the three experiments, has recently been demonstrated in apple (8). The confinement of effects to the chilled tissue is also characteristic of rest-breakage (3, 17) and vernalization (15).

Translocated hormones have been found to induce or inhibit acclimation in several broadleaved species as cited above. However, an alternative explanation of the present results could be based on nutritional requirements. In Experiment 2, the warm branch might simply have supplied additional photosynthate to the rest-broken bud of its partner thereby permitting the partner to flush even though the partner's own carbohydrate production was curtailed by an insufficiently long warm-day (8 hr). The expanding shoot might then continue to act as a sucrose sink, causing a nutritional dehardening in the warm donor, of the type that occurs during prolonged darkness (12). There are several points against this interpretation. (1) Foliage on the chilled branch bearing the new flush did not lose hardiness, yet there is no reason to suppose that it was any less prone to deharden as a result of the putative carbohydrate demand (without making further, complicating assumptions). (2) In Experiment 3, flushing proceeded just as rapidly (also under an 8-hr day) in spite of the absence of a warm partner, indicating that no additional photosynthate was necessary. (3) The lower hardiness of chilled branches with warm partners, when there was no flushing, is difficult to explain on any source-sink nutritional basis, but on a hormonal basis this can be attributed to the same factor that the warm branch supplies to promote flushing. (4) The photosynthetic requirement for maintaining hardiness is small

(sufficient was produced under a 200 ft-c 8-hr photoperiod at 2°C in earlier studies, 18), and the period of negative CO_2 uptake in flushing shoots is relatively brief (13). Thus, photosynthesis in old needles would probably be adequate for both. These four objections strongly favour the hormonal hypotheses.

The data indicating a warm-night flushing promoter sequential to chilling, are not conclusive, because it was not known for certain (1) whether the absence of a root temperature effect on hardiness (Experiment 1) and on flushing (Experiment 3) was also true for the interior provenance used in Experiment 2; or (2) whether the 29-day continuous chilling requirement for the Cranbrook provenance (17) had been satisfied by the 130 days of intermittant chilling actually given. With regard to the latter uncertainty, Bennett (2) found that the chilling requirement of pear buds was increased by alternating warm and cold temperatures. If, as Bennett's data imply, there were a warm-day reversal of nightly chilling, then flushing promotion in split plants could be attributed, for example, to some continuing effect of the warm-night factor in preventing this reversal. A more probable explanation in the absence of adequate chilling is that the warm branch might simply have supplied a long photoperiod hormone which, in partially rest-broken plants, is often necessary to provide the added stimulus for growth (14). The photoperiod effect may not have been perceived by the cold-night branch due to

retardation of phytochrome or intermediate dark reactions; earlier results (18) showed an absence of SD-induced bud setting below 15°C. However, the substantial chilling of 15 hr/day for 130 days makes explanations based on incomplete rest-breakage seem improbable. The remaining alternative to the original hypothesis, namely that long photoperiods are necessary even after rest has been fully broken, finds little support in the literature, and would be in disagreement with the results of Experiment 3. In terms of the current idea that an inhibitor-promoter balance controls the breakage of rest (1, 16), the present results suggest that the levels of these two regulators might be controlled independently. Perhaps they also act at separate biochemical sites rather than in quantitative balance.

The dehardening during expansion of new shoots occurred in both provenances. Irving and Lanphear (11) found that breaking rest in Acer negundo and Viburnum plicatum accelerated subsequent dehardening. They reviewed similar findings by others, but apparently none of these studies reported on changes associated specifically with flushing. In Howell and Weiser's data for apple (9) significant dehardening patterns did accompany the phenological changes, although it is not possible to distinguish these entirely from natural environmental temperature effects. Worrall (17) has shown (using the Cranbrook provenance and similar temperature regimes) that flushing of previously chilled buds leads to cambial

activity in lower unchilled regions of the stem, in accordance with the theory of polar auxin transport. However, the loss of hardiness in warm foliage of branches with flushing partners cannot be attributed to the same factor because no new xylem was formed in these branches. Therefore the "close coincidence" of cambial activity with gain and loss of hardiness in this species (5) does not have a common hormonal basis.

The initial dehardening in Douglas-fir occurs in response only to temperature (4). The present results suggest that final loss of hardiness (above the short-day induced level) is dependent on factors from expanding shoots. The data for apple (9) appear to embody both influences. Such a dual control of dehardening would be ecologically important. While the cold-induced (and deeper) phase of hardiness (18) may fluctuate rapidly in response to short-term changes of temperature (16), the SD-induced phase might impose an upper limit on unseasonal dehardening until, as a result of cumulative longer-term environmental changes, the plant is committed to its summer programme of growth.

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CHAPTER 4

ELECTRICAL AND THERMAL RECORDS OF FREEZING IN DOUGLAS-FIR NEEDLES

ABSTRACT

The progress of freezing in needles of hardy and nonhardy branches of climatically "split" Pseudotsuga menziesii (Mirb.) Franco seedlings was recorded simultaneously by differential thermal analysis and the conductance of low voltage direct electric current. The results of both methods exhibited the same major patterns. Freezing in immature leaves was nonequilibrium and intracellular. Freezing in needles cold-acclimated under short days was an equilibrium process preceded by a short non-equilibrium freezing of the free intercellular water fraction. This pattern did not change in leaves more deeply cold-acclimated by low temperatures.

Thawing in mature needles was characterized by a greater proportion of ice (than during freezing) at all temperatures, with indications that not all the original cell water was reabsorbed. Freezing records are interpreted as showing that the cell membrane became more permeable to ions after injurious slow freezing but retained its essential integrity, whereas rapid freezing caused immediate membrane damage. No features of the freezing or thawing curves of first or subsequent freeze-thaw cycles were useful as predictors of injury by slow freezing. The proportion of unfrozen water, determined calorimetrically, was

less in immature needles and did not differ between hardy and nonhardy mature ones, but the latter data are inconclusive.

INTRODUCTION

Most theories on cold hardiness accord some role either to changes in cell membrane permeability (17, 18, 24), water binding (18, 38, 42), type of extracellular nucleators and crystal growth (30), loci of ice nucleation with respect to tissues (1, 9, 11, 31), or to other adaptations reflected in the overall kinetics of ice formation in whole tissues or organs. While there are definite limits to the resolution and interpretation of measurements made at this level (18 27), such records nevertheless provide valuable information for a species under initial study.

Two main methods have been developed for recording ice formation without the disturbance of microscopic or macroscopic sectioning. These are measurement of heat of fusion, and monitoring the conductance of weak direct or low frequency electric currents. There is considerable uncertainty in the interpretation of each. Exothermal profiles tend to be distorted according to thermocouple placement and smoothed out due to heat conduction lags (40). It is always difficult to distinguish tissue-by-tissue compartmentalization of the freezing process from the freezing of particular water fractions within a tissue (1, 33). The characteristic double freezing point has not been satisfactorily explained (23), and respiration during thawing may contribute significant amounts of heat (16). It is generally accepted that low frequency electric current is conducted through

the apoplast of healthy tissues because of the relatively high resistance of the cell membrane (3, 7, 28). Thus, conductance as a function of temperature under model conditions has been considered to represent the proportion of unfrozen extracellular water in equilibrium with extracellular ice and with supercooled cell water (28). Stamm (34) notes, however, that conductance of water in wood fibre walls actually decreases at a faster-than-linear rate with mass. In living tissues during freezing and thawing the conductance represents, in addition, the cumulative effects of: (1) possible membrane permeability changes due to non-injurious chilling (9, 10, 14, 18); (2) partial irreversibility of water efflux (6, 35) from the cells during freezing; and (3) injurious changes in membranes during freezing, while frozen and during thawing (5, 19). For both types of measurement equilibrium at any given temperature is probably not attained under simulated frosts of fairly short duration on intact plants (16), and random supercooling effects determine the onset and initial level of the "steady state" which is recorded instead. Because of these uncertainties, both electrophoretic and thermal records were obtained from a sub-sample of replicates in the present experiment to improve confidence in the largely qualitative comparisons.

Application of both these techniques to potato leaves (36) showed differences between hardy and nonhardy varieties which

indicated earlier membrane injury in the nonhardy type. In Douglas-fir seedlings differing markedly in hardiness, no clear differences in electrophoretic freezing patterns (e.g., of the type reported by Olien in barley crowns, 29) were observed (39). However, genotype and water content variation was considerable in these preliminary studies, and may have obscured differences actually present.

The aims of the present study were: (1) to establish typical freezing patterns in immature and in hardy and nonhardy mature needles by the two methods; (2) to extend this comparison to the thawing process and to repeated freeze-thaw cycles for observation of reversibility and the effects of injury; and (3) to compare thermograms of excised needles obtained by differential scanning calorimetry (DSC) with the in situ exotherm records, and to obtain quantitative estimates of the mass of ice.

MATERIALS AND METHODS

Plants

Two-year-old Pseudotsuga menziesii (Mirb.) Franco seedlings of a coastal provenance (500 m, Duncan, Vancouver Island) were used in all experiments except DSC, which was conducted on an interior provenance (Cranbrook, B.C.). Seedlings were raised in a greenhouse and pruned at the beginning of the second growing season to produce forked tops. In August the plants were acclimated in a cold room at 5°C with one branch in a plexiglass

chamber at 20°C. Night temperatures were 2°C lower in each case. Both chilled and warm branches received 500±50 ft-c mixed incandescent and fluorescent light 8 hr/day for 6 weeks. This regime produced an average difference of 12°C in hardiness in the foliage of warm and cool branches (41). One day before sampling for freezing tests, pots were soaked with water and the warm chamber humidified to bring foliage to a comparable state of hydration. Immature foliage was obtained from warm branches which had produced a late flush.

Measurement of Injury and Hardiness

The foliar hardiness of individual branches was first established by sequential freezing tests on excised mature needles. Injury was assessed visually on a 10-point scale after a 7-day recovery period and hardiness was defined as the temperature corresponding to 50% injury (40). In addition, injury to unmonitored samples, frozen alongside monitored needles, was assessed for each particular run as a check on electrode injury.

Freezing

Methanol coolant was pumped through a dry ice/acetone bath, and temperature control was achieved by reheating in a Dewar flask reservoir under the control of a commercial temperature programmer. The coolant circulated slowly through vinyl tubing surrounding a freezing chamber equipped with an internal fan. Uniform cooling and warming within a wide range of rates and

temperatures could be achieved and duplicated in successive cycles by this arrangement. Freezing curves described below are based on a pre-determined maximum safe cooling rate of 7°C/hr, followed by warming at 20°C/hr with a 15 °min period at the minimum temperature, unless otherwise stated.

Recording of Ice Formation

Gold plated copper electrodes were placed approximately 1 cm apart on the needles, and partially encircling them. Pastes of charcoal for electrical contact (28) proved unreliable on these relatively xerophytic leaves and a commercial electro-conductive paste of electrolytes was used sparingly instead (EKG Sol, pH 5.6, Burton, Parsons and Co. Inc.). The localized injury which resulted caused a gradual increase in the current flowing at constant temperature, but only at temperatures above freezing. Under isothermal conditions after initial freezing, the current flowing at 3 v remained steady over long periods of time in the microamp range. The paste does not freeze in the temperature range investigated, and it is assumed that its change in conductance with temperature is adjustable by the same viscosity correction factor as that for pure water (see below).

The freezing chamber contained six sample holders and electrode pairs, each linked in series with a dry cell and strip chart recorder, permitting three hardy and nonhardy replicates to be monitored at once. One of the replicates from each treatment

was also monitored by a 30-gauge thermocouple with its reference junction at ambient air temperature to provide a differential thermal profile. The arrangement for a single sample is shown in Figure 1. Subsequently, tests were performed on detached needles because it was found that these underwent freezing in a similar manner and without difference in injury, providing that water was supplied to the basal end from the small reservoir via a filter paper wick. This permitted numerous comparisons within a single plant.

The time-based record of current flowing through the sample was corrected for temperature and initial current, and the relative mobility of liquid water (28), M, plotted as a function of temperature, during freezing and thawing.

$$M = \frac{I_t}{I_o} \times \frac{V_t}{V_o}$$

where I_t and I_o are current flowing at t and 0°C respectively, and V_t and V_o are the viscosities of liquid water at these temperatures. Viscosity values below -10°C were extrapolated from tabulated values (38) using the empirical relation $V_t = Ae^{B/RT}$ where T is the absolute temperature, R the gas constant, and A and B constants for the fluid. A and B are assumed not to change with the degree of colloidal association of water with extracellular surfaces.

Calorimetry

The DSC cell of the Dupont 600 Differential Thermal Analyser

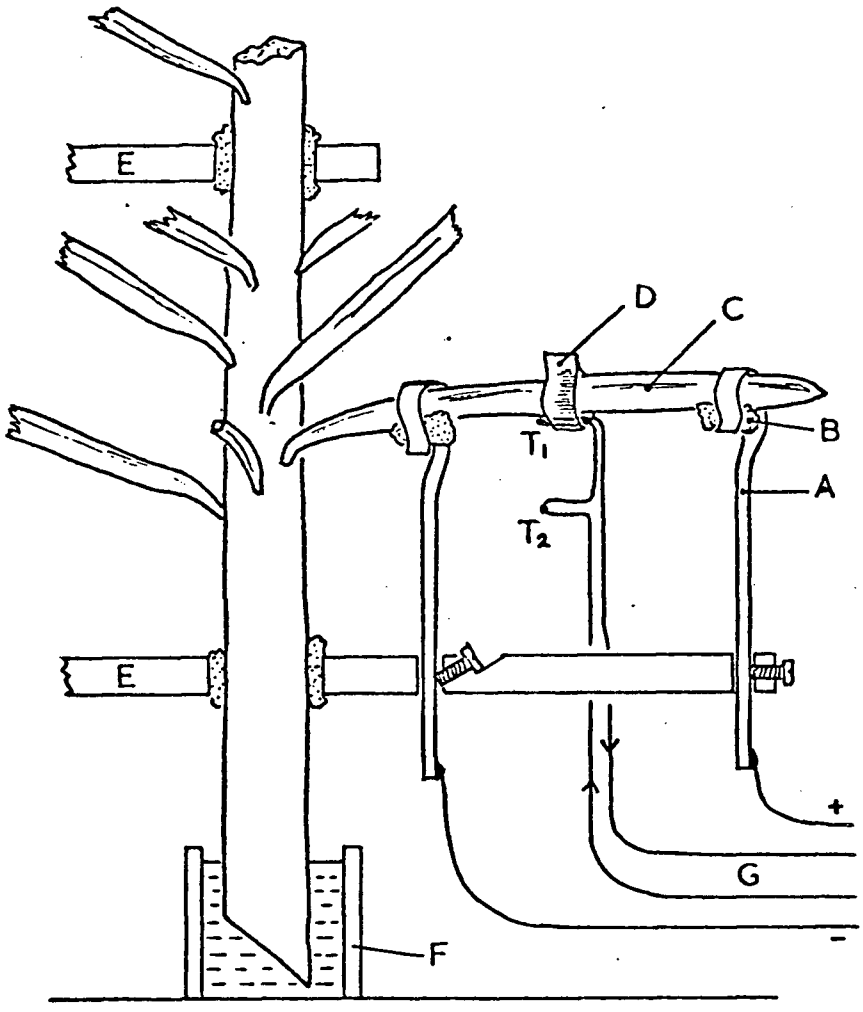

Fig. 1. Arrangement for obtaining thermal and electrophoretic freezing records of needles. Attached needle (C) is held into gold plated electrodes (A) by small piece of foam rubber (B). Thermocouple junction (T_1) is held against lower surface of leaf by a light adhesive tape (D), and records temperature difference from ambient (T_2). Electrode and thermocouple leads (G) are input to an external dual-channel recorder. The twig is held in an supportive framework (E) with its base in water (F) inside the freezing chamber.

was operated at its slowest cooling rate of 30°C/hr with liquid nitrogen as coolant. A weighed (±0.025 mg) needle was placed, partly coiled up but undamaged, in the sample pan, while the reference pan remained empty. An external time-based strip chart recorder recorded the exotherm during freezing. Oven-dry weight was obtained after 24 hr at 105°C. A total of 42 runs was made on the hardy and nonhardy needles of 10 plants.

Amount of water frozen, W, at completion of detectable exotherm was calculated from:

$$W = \frac{A.\Delta T.E}{\Delta H.C}$$

where A = area under peak (cm^2), ΔT = sensitivity of recorder setting (°C/cm), E = calibration factor (calories/°C-min), ΔH = latent heat of fusion of pure water (cal/gm), and C = chart speed (cm/min). W was expressed as % of total water at full turgor, measured gravimetrically. E was determined from runs using pure water or mercury for which both W and ΔH were known. No attempt was made in this comparative study to correct for several sources of error (41), which are assumed to affect both treatments more or less equally.

RESULTS

Freezing Curves

Freezing of immature needles was invariably a nonequilibrium and lethal process characterized by a single sharp exotherm and a

sudden decrease in M (Fig. 2). Simple hand sections made after thawing showed rupture of cells and release of their contents, clearly indicating intracellular freezing. In about half of the trials, including a number made on succulent stem tissue of 4-week-old seedlings in a separate investigation, the decrease of current was immediately preceded by an increase of 0.5 to 2 sec duration (Fig. 2).

Mature leaves exhibited, in most cases, both nonequilibrium and equilibrium freezing processes corresponding with the first and second exotherms respectively. A derivative plot, $\frac{dM}{dT}$ (Fig. 2) indicates that the electrical measure records the first freezing with much greater sensitivity, as would be expected if this represents the free extracellular water.

No differences were detectable between the freezing curves of hardy and nonhardy mature needles, even though genetic variation had been avoided by the use of split plants. Figure 3 illustrates the scatter among six hardy and nonhardy needles of a single plant. Other plants were equally variable.

Thawing

A hysteresis was consistantly observed in both electrophoretic and thermal records of a freeze-thaw cycle (Fig. 4). Thawing was a gradual and continuous process producing a single smooth endotherm. It appeared that even under very slow cooling or warming rates, or when temperatures were held constant to

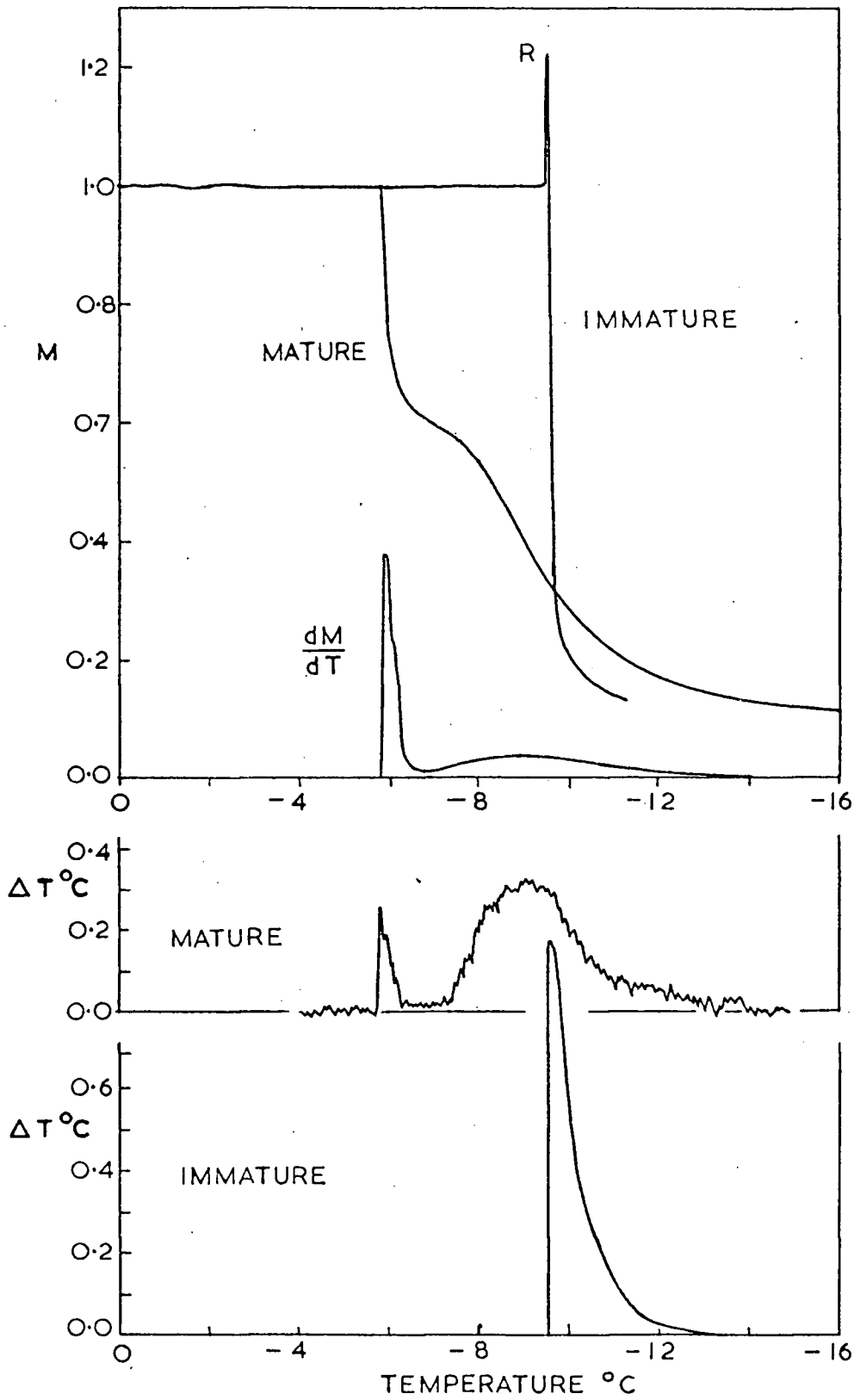

Fig. 2. Typical records of relative mobility of water, M (see materials and methods), and exotherms, during freezing of mature and immature needles. ΔT = elevation of leaf surface temperature above ambient. dM/dT is the derivative of the mature-needle M curve with respect to temperature (arbitrary units). Peak R was observed in about half the trials. Cooling rate was 7°C/hr.

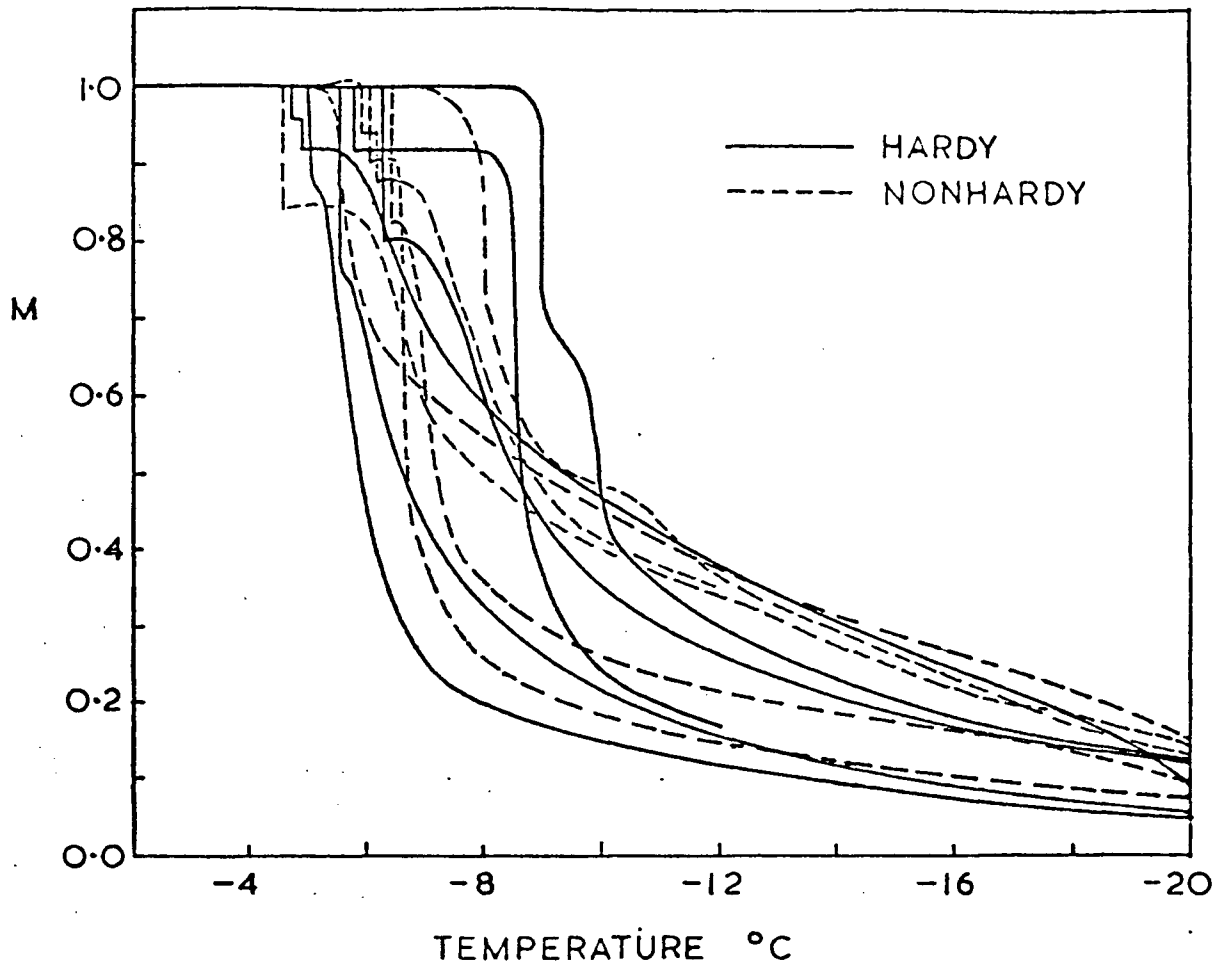

Fig. 3. Relative mobility of water, M (see materials and methods), as a function of temperature, in hardy and nonhardy needles of a single split plant. The observations were made under comparable conditions within a 2-day period. Cooling rate was 7°C/hr.

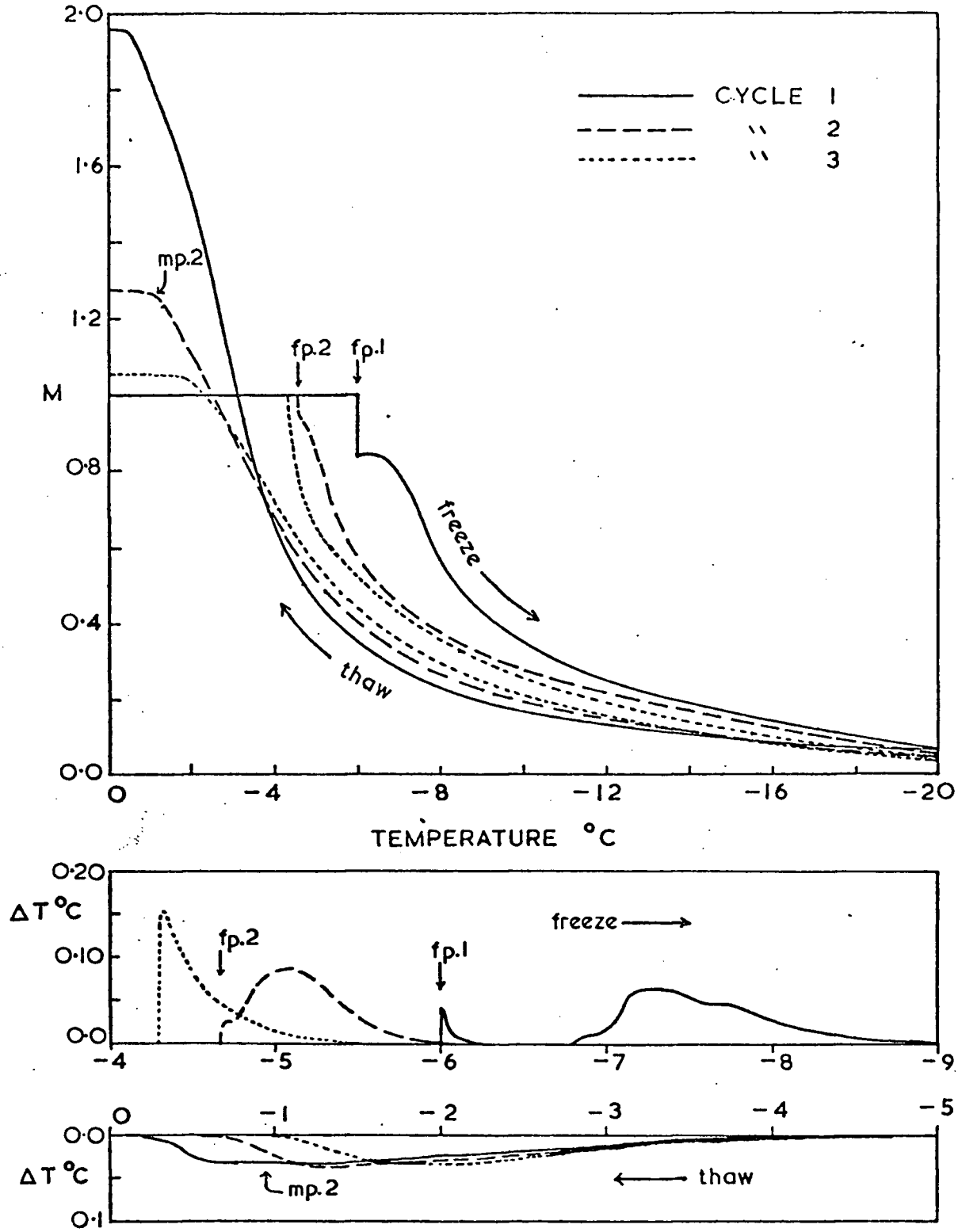

Fig. 4. Relative mobility of water, M (see materials and methods), and temperature elevation/depression (ΔT) in mature needles during 3 cycles of freezing and thawing. The diagram is based on average values of the various onset and inflection points from 14 trials, and on actual curve shapes. fp = freezing point, mp = melting point. Cooling and warming rates were 7°C/hr and 20°C/hr respectively.

allow closer attainment of equilibrium, the proportion of ice at a given temperature was higher in the thawing cycle. However, final values of relative mobility (M_F) always exceeded pre-freezing values. M_F was significantly higher when mean values of nonhardy needles in a run were compared with hardy means (Table I), but no correlation could be established between M_F and injury to individual needles assessed after 7 days. After injury by rapid freezing to a normally safe temperature, on the other hand, or after injurious warming rates, M_F and visual injury followed a similar trend (Fig. 5).

Repeated Freeze-Thaw Cycles

Triple cycles were monitored on seven replicates of hardy and nonhardy needles from two seedlings. M was calculated with respect to I_o at the beginning of the cycle in question.

Exotherms show a progressive loss of "structure" and their relative sharpness indicates a more rapid initial crystallization during successive frosts in spite of significantly reduced supercooling. These changes are also noticeable in the electrophoretic records which, in addition, show a progressive reduction in M_F and a decrease in the upper melting point. Hysteresis tends to become less pronounced. These features are shown schematically in Figure 4 and individual values of significance are given in Table II.

Table I. Relative increase in Conductance (M_F) of Weak Electric Current Through Douglas-fir Needles After Slow Freezing

Run No.	Tree No.	Hardy Branch: Mean Killing Point of Needles	Hardy Branch: M_F	Nonhardy Branch: Mean Killing Point of Needles	Nonhardy Branch: M_F
1	1	-28.0	2.03 (2)	-15.5	5.51 (2)
2	1	-28.0	1.96 (3)	-15.5	2.32 (3)
3	2	-31.0	2.95 (2)	-15.5	4.78 (2)
4	3	-26.5	1.48 (2)	-16.5	2.34 (2)
5	3	-26.5	1.63 (3)	-16.5	1.70 (3)
6	3	-26.5	1.74 (2)	-16.5	1.75 (2)

Note: Numbers in parentheses indicate number of replicates of which M_F is the mean. Killing points were obtained by sequential freezing tests in preliminary experiments using larger samples.

The low temperature was -20°C (except -12°C in run No. 6).

M_F values of hardy and nonhardy populations differ significantly at the 5% level.

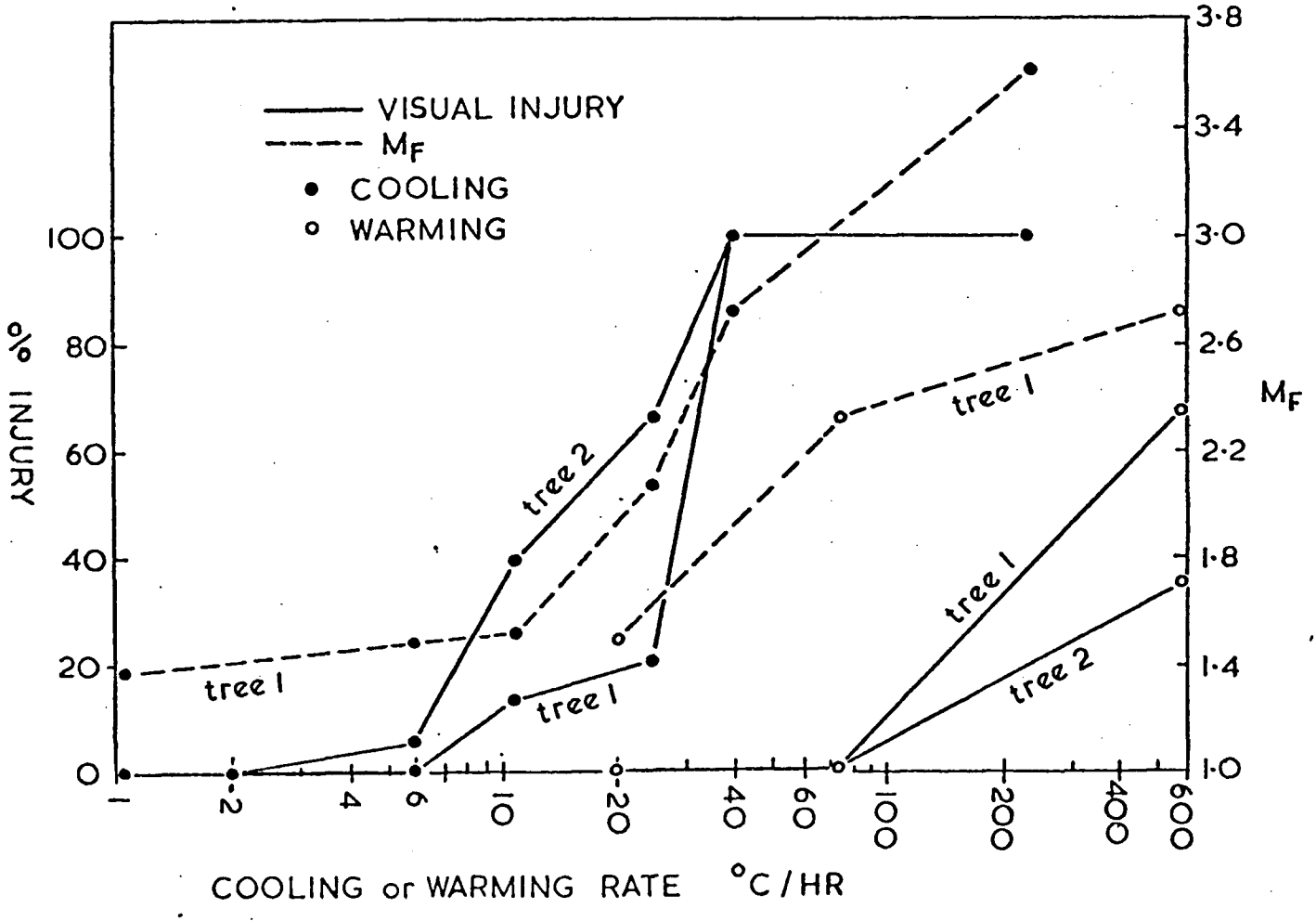

Fig. 5. Freezing injury to hardy needles as a function of cooling and warming rates (logarithmic scale). M_F = relative mobility of water (see materials and methods) after thawing. Injury was assessed visually after 24 hr as the proportion of browning in two monitored needles (as Fig. 1) plus 5 others from the same twig. Killing temperatures for foliage of trees 1 and 2 were -21 and -25°C respectively at natural cooling and warming rates. Cooling rate trials were warmed at 20°C/hr; warming rate trials cooled at 7°C/hr. The low temperature was -18°C in all cases.

It is reasonable to suppose that changes in successive cycles are mainly due to injury. Table II compares these parameters with respect to hardiness and visually assessed injury. Second and third cycle melting points were significantly lower in the nonhardy group. Also, M_F approaches unity sooner (that is, after fewer cycles) for nonhardy plants, reflecting the fact that in these the point of maximum injury is more rapidly attained. The loss of the second exotherm in subsequent cycles, however, was not consistantly associated with corresponding injury, nor even with the hardiness class, except where rapid freezing (Fig. 5) had been the cause of death.

Calorimetry

Freezing of detached needles in the DSC cell took place in many cases at temperatures well below the normal supercooling range. Supercooling was highly correlated with various parameters describing exotherm shape and with percentage of total water frozen at completion of the detectable exotherm, but was itself a largely random event. Lower freezing temperatures were significantly correlated with lower tissue water contents to a small extent, (R^2=0.11).

Of those runs which fell within the supercooling range of freezing chamber studies, exotherm shape resembled that recorded by a thermocouple at the surface of attached needles. The first exotherm represented between 5 and 15% of the final mass of ice.

Table II. Characteristics of Electrical Conductance Records of Douglas-fir Needles During Repeated Freeze-thaw Cycles in Relation to Hardiness and Injury

Repl. No.	Tree No.	Branch	Supercooling °C. Cycle 1	Supercooling °C. Cycle 2	Supercooling °C. Cycle 3	Melting point °C. Cycle 1	Melting point °C. Cycle 2	Melting point °C. Cycle 3	M_F Cycle 1	M_F Cycle 2	M_F Cycle 3	Visual % injury 1 day after cycle 3
1	1	H	5.0 D	3.4 I	3.2 I	-0.2	0.0	-1.0	1.70	1.33	1.12	15
		NH	6.4 D	4.7	4.4	0.0	-1.2	-3.4	1.83	1.19	1.02	100
2	1	H	4.8 D	4.2 I	4.1	-0.2	-1.0	-1.0	1.93	1.24	1.08	18
		NH	6.0 D	4.2 I	4.0	-1.2	-1.5	-2.4	1.48	1.12	1.05	100
3	1	H	5.5 D	3.5	3.4 I	-1.1	-1.0	-1.0	2.24	1.12	1.11	13
		NH	5.3	3.3	4.0	0.0	-1.2	-2.7	3.66	1.43	0.90	100
4	3	H	6.4 D	5.0 I	4.6	0.0	-0.5	-2.2	1.48	1.33	1.11	58
		NH	4.6 D	5.0 D	5.5	0.0	-1.3	-2.7	1.86	1.35	1.00	100
5	3	H	8.6 I	5.6 I	4.6	-0.6	-1.0	-0.8	1.48	1.13	1.00	62
		NH	7.4	4.7	4.0	-1.0	-1.0	-1.2	2.83	1.42	1.03	100
6	3	H	5.8 D	5.0 D	5.0	-0.5	-1.0	0.0	1.64	1.28	1.09	64
		NH	6.2 D	5.0	4.8	-1.2	-2.0	-1.5	1.28	1.21	1.00	100
7	3	H	5.8 D	5.3	5.0 I	-0.2	0.0	-1.4	1.84	1.24	1.20	62
		NH	5.9 D	4.7	4.3	-0.2	-1.0	-1.2	2.22	1.45	1.00	100
Mean		H	6.0	4.6	4.2	-0.40	-0.64	-1.06	1.76	1.23	1.10	41
		NH	6.0	4.5	4.4	-0.51	-1.31	-2.16	2.16	1.31	1.00	100

Note: Letters after numerical values indicate presence of distinct (D) or indistinct (I) second exotherm, in hardy (H) and nonhardy (NH) needles.

M_F is the relative increase in conductance after thawing compared with that at the beginning of the cycle. Injury values represent the mean of the monitored needle and 5 unmonitored needles.

In immature needles 85 to 95% of the water froze, whereas in mature needles 40 to 75% solidified (Table III depending partly on supercooling. When individual values were covariance-adjusted for supercooling, there were no significant differences between the proportions of unfrozen water in hardy and nonhardy mature leaves. Means are shown in Table III.

DISCUSSION

The intolerance of ice formation by immature tissues, due to intracellular freezing, has been widely reported for other species (8, 11, 29, 35) and appears to be the case in the present study. It is improbable that 90% of the water could have moved so quickly, and without any exothermal discontinuities, to sites outside the cell. It is further considered that the brief increases of current, registered only by recorders with a relatively fast response time, are probably equivalent to the somewhat slower ones observed in tender tissues of barley (28, 29). This would mean that they represent the momentary situation where ice crystals have ruptured the cell membrane, but have not spread completely throughout the cell. The probability of intracellular nucleation is greater in immature tissues, which have a higher cell water content, a relatively small volume of intercellular space, a lower membrane permeability (18) and a lack of effective extracellular nucleators (12). In support of this, the range of supercooling of immature versus mature Douglas-fir tissues was generally farther below 0°C.

Table III. Percent Frozen Water at Completion of Detectable Exotherm in Hardy and Nonhardy Excised Douglas-fir Needles Cooled at 30°C/hr in a Differential Scanning Calorimeter

Tree No.	Mature Hardy Mean Killing Temp.= -24°C		Mature Nonhardy Mean Killing Temp.= -11.5°C		Immature Mean Killing Temp.= -6.5°C	
	Super-cooling °C	% Water Frozen	Super-cooling °C	% Water Frozen	Super-cooling °C	% Water Frozen
	11.2	56.8	10.0	46.4	....	....
	10.7	62.6	14.2	64.2	....	....
	13.7	54.5	20.0	68.5	20.0	94.2
	14.5	63.4	15.5	74.7	19.5	85.8
	7.0	55.2	8.0	50.6	....	....
	14.7	53.9	11.7	51.7	....	....
	7.5	46.9	7.7	40.0	....	....
	12.2	49.9	15.5	60.2	14.3	87.0
	10.7	47.2	12.2	44.0	....	....
	13.5	46.8	13.7	56.5	....	....

Note: Values represent the mean of two determinations made on an interior British Columbia provenance (Cranbrook, B. C.).

However, after the change from nonequilibrium to largely equilibrium freezing associated with maturation and preliminary cold acclimation under short days, no further changes in the electrophoretic "freezing curve" accompanied the additional acclimation which occurs at low temperatures. McLeester et al (22) report two or three exothermal freezing curve shapes in red osier dogwood twigs passing through a similar hardiness range, but over a period of time. The evidence that the nonequilibrium part of the mature needle freezing curve is due to free extracellular water supports conclusions made by other workers (reviewed in 23).

The hysteresis is considered to be at least partly an actual characteristic of the living colloidal system rather than a simple lag effect due to failure to attain equilibrium at a given temperature. The evidence for this is that: (1) the hysteresis is not sensitive to cooling and warming rates within a wide range; and (2) it tends to become less pronounced as injury increases in subsequent freeze-thaw cycles. Stark (33) first noted a greater amount of ice in the thawing part of a cycle using calorimetry. More recent data on yew and holly leaves (42) support this, and show that equilibrium, after a sudden 10°C temperature change in thawing, is closely approached within only 8 min. The time allowed for an equal temperature change in these studies was several times greater. Krasavtsev (16) has attributed this "general imbalance of heat exchange" to respiration

during thawing. The present electrical conductance measurements, however, may be considered insensitive to respiration effects. The fact that M_F exceeds unity, even without injury, indicates that not all the original cell water is re-absorbed from the intercellular space. This is another aspect of the hysteresis, but one which needs further verification due to the possibility of membrane permeability transients cited earlier, and significant electrode effects.

Features of freezing and thawing curves offer attractive possibilities for quickly estimating injury, and hence hardiness, in physiological studies. Glerum (4) mentions many early investigations showing that electrical conductance increases after stress. He found that conductance of conifer twigs increased 76 to 93% after lethal freezing, and Takeda (36) obtained a correlation between the actual degree of frost and subsequent low frequency conductance in needles of Cryptomeria japonica (L.) Don. The value of M_F obtained in the present study, however, while differing on average between hardy and nonhardy populations, is not a suitable predictor of partial injury. This suggests that injury from equilibrium freezing at stages of moderate acclimation arises from causes other than simple membrane damage, and takes some time to develop. This is a conclusion also postulated by Evert and Weiser (2). It is further strengthened here by the observation that injury by rapid freezing, generally regarded as an

intracellular process (24), produces an immediately detectable and proportional increase in M_F (Fig. 5). It is also in agreement with the results of an earlier study (40) which showed that Dexter's electrolytic measurement of freezing injury, based on the leakage of ions through injured membranes into an external solution, is less sensitive than visual estimation of browning after 7 days. The unsuitability of multiple freezing points during refreezing as a criterion of viability is also in contrast to other work (23), and possibly for the same reason. However, interpretation is complicated by the appearance of lower melting points in subsequent cycles, especially of nonhardy leaves. Since these decreases in melting point are not accompanied by large increases in M_F, e.g., between the second and third freeze-thaw cycles, then it is presumed that they represent a leakage of ions into the intercellular space and a depression of freezing point there, rather than a traversal of electric current through the relatively concentrated solution of the cell interior. Simple osmotic pressure differences between hardy and nonhardy cells (cited by Levitt, 18) would tend to act in the opposite direction, and in any case are mainly due to non-electrolytes (32). Thus, the tentative conclusion is that the cell membrane becomes leakier during injurious freezing but still retains its integrity as a barrier to electric current and ice crystal growth, while critical injury proceeds as a comparatively slow development of some "secondary plastic strain" (20).

Postulated irreversible strains of freezing include removal of "vital" water (13, 40), salt-denaturation of proteins (21), and mechanical effects following dehydration below a certain minimum volume (25, 26). These are avoided if the cell is able to retain a sufficient amount of water in the liquid state. However, this method of avoidance by hardy tissue is not apparent from the data of Table III. Meyer (27) also detected no difference in amount of unfrozen water between hardy and nonhardy mature Pinus resinosa Ait. needles, but in the majority of early investigations on herbaceous plants, cited by Levitt (18), the amount of ice in hardy tissues at a given temperature was significantly less. More recent and precise work (15, 16, 38) confirms, for apple twigs, the latter result within the temperature range of interest here. Because of the unnaturally high cooling rate, compounded by erratic and excessive supercooling, and other technical limitations of the commercial instrument used, the calorimetric measurements in this work are regarded as inconclusive. In those cases where abnormal supercooling, and probably extensive intracellular freezing, did not occur, the data indicate that there were no major differences in the proportion of unfrozen water between hardy and nonhardy leaves.

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CHAPTER 5

THE ROLE OF BOUND WATER IN COLD HARDINESS OF DOUGLAS-FIR NEEDLES

ABSTRACT

Binding of water in climatically split Pseutosuga menziesii (Mirb.) Franco seedlings was compared for mature needles differing in hardiness by 25°C. Heats of vapourization (ΔH_v) of weighed increments of water, removed from excised needles under vacuum, were estimated from the calibrated vapourization endotherms recorded on a differential thermal analyser. In a second method, needle water contents were measured gravimetrically after equilibration with lithium chloride solutions of known desiccating energy.

ΔH_v, a proposed measure of water binding near surfaces, increased as the proportion of water remaining in the leaf decreased. For each increment removed, ΔH_v was significantly higher in hardy needles, notwithstanding various possible sources of error. Hardy needles also retained more water non-osmotically (than nonhardy needles) after equilibration with LiCl isopeistic with their frost-killing temperature. Dehydration avoidance probably accounted for 12 centigrade degrees of the hardiness difference between branches.

INTRODUCTION

The most injurious stress in hardy plant tissues during slow freezing is the dehydration of cells as their water moves to intercellular ice crystals (11). The dehydrating force may be expressed as the free energy difference for vapourization.

$$\Delta F_v = RT \ln \frac{P}{P_o} \qquad (1)$$

where R is the gas constant, and where P is the vapour pressure over ice and P_o that over supercooled water, both at absolute temperature T.

Levitt (11) has emphasized that cells may survive freezing either by being able to tolerate the dehydration in some manner, or by retaining water against the dehydrating force (dehydration avoidance). This water retention or "binding" may be achieved to a limited extent by an increase in the solute content of the cell water; it may especially be due to an increase in the number or nature of internal surfaces which impose some short range order and stability on the complex water structure (2,13).

Osmotic retention is only able to protect against light frosts because the ΔF_v of physiologically high concentrations of, for example, sucrose (which can be calculated from Equation 1 taking P as the vapour pressure over the solution and P_o as the v.p. over pure water, both at temperature T) is equivalent to that of extracellular ice at only a few degrees below 0°C. A maximum equivalence of about -15°C is possible, for a saturated solution, but this would be achieved only in very

dehydration-tolerant cells during the freezing process. Sukumaran and Weiser (24) have shown that the osmotic potential of potato leaf cells is sufficient to account for the greater (but still relatively low) hardiness of certain varieties.

Water bound at and near surfaces, on the other hand, "probably has a continuous spectrum of activation energies" (13), and is quantitatively retained at the very lowest temperatures occurring in nature. A number of investigators have measured this fraction, between arbitrarily defined binding energies in hardy and nonhardy plants, to determine its role in freezing tolerance. These studies have given different results according to the species and method used.

The earlier investigations have been critically reviewed by Levitt (9), and indicated in most cases that there is a greater proportion of non-osmotically bound water in hardy cells (exceptions included conifer leaves). Levitt (10) used a vacuum and heating method to differentiate between free and bound water in various cell fractions of cabbage. He found significantly more bound water in hardened than in unhardened plants. However, Parker (18) detected no seasonal differences in white pine and eastern red cedar leaves with the same method. Studies on winter wheat (20,21) have shown that hardened tissues retain more total water than unhardened tissues when at equilibrium with aqueous solutions of NaCl, but in this case the non-osmotically bound portion is derived by making the unjustified assumption that this surface-binding is a special energy-requiring characteristic of the living cell. Calorimetric

measurements of hardy and nonhardy wheat and apple (7,29) show that the proportion of "bound" (i.e. unfrozen) water is greater in the hardy plants over a range of freezing temperatures, but also make the same invalid assumption about surface binding.

In general, where the data permit conclusions about the temperature or dehydration-tolerance ranges over which surface binding confers protection, these conclusions are usually that such binding is insufficient to fully account for the observed hardiness differences. Nevertheless, any avoidance of dehydration stress by this means would contribute quantitatively to the overall freezing tolerance.

The present study was undertaken to determine the role of cell water retention in frost tolerance of Douglas-fir needles. Two methods were employed to measure the energy of water in leaves. In one method the heat absorbed during the vacuum-evaporation of a known mass of water from intact leaves was estimated by means of a differential scanning calorimeter (DSC). In the other, the water content of leaves at equilibrium with lithium chloride solutions of known dehydrating energy was determined gravimetrically. Both methods are described below.

MATERIALS AND METHODS

Plants

In order to avoid genotypic variation and reduce variation in calorimetric data associated with leaf mass and shape, needles were taken from hardy and nonhardy branches of the same plant. To obtain these, two-year-old Pseudotsuga menziesii (Mirb.) Franco seedlings of a

coastal provenance were differentially cold-hardened by a procedure in which one branch was subjected to chilling and night frost, while the other was made to deharden and grow in a warm environment, as previously described (28). This treatment resulted in needles of warmed and chilled branches having a hardiness of approximately -10°C and -35°C respectively. Hardiness was defined as the freezing temperature causing 50% visible injury to excised needles (26).

Theory of the Calorimetric Method

There is evidence that the proportion of structurally modified water in living cells is considerably more than that accounted for by hydrogen-bonded monolayers, and will increase the average heat of vapourization by a measurable amount. The highly bound single layers themselves may constitute up to 50% of the dry weight (2), yet the influence of hydrophylic surfaces generally extends through two or three water molecules (2,8,15,23). Furthermore, "hydrophobic bonding" is thought to be responsible for stabilization of a large proportion of water as "icebergs" contained by non-polar surfaces (2,25). Nuclear magnetic resonance spectra have indicated at least two phases of "ordered" water in some tissues (4). Drost-Hansen (2) has cited evidence that nearly all the water in tissues has a greater or lesser degree of structural ordering imposed upon it.

The extent to which water binding will increase the latent heat of vapourization can be calculated, and compared to that of water in bulk, if we know how the vapour pressure of bound water varies with its

temperature. These factors are related by the Clausius-Clapeyron equation, which in its integral form is:

$$\Delta H_v = R \frac{T_1 T_2}{T_2 - T_1} \ln \frac{P_2}{P_1} \qquad (2)$$

where ΔH_v is the heat of vapourization (cal/mole), R the gas constant (cal/mole/°C), T_1 and T_2 are two absolute temperatures, and P_1 and P_2 the respective vapour pressures. The presence of solute molecules, even at high concentrations, does not affect the slope, $\frac{dP}{dT}$, of the vapour pressure/temperature curve, and therefore ΔH_v is not affected. But $\frac{dP}{dT}$ is affected by surface binding (5,23). Vapour pressures of water bound in wood fibres are given by Kelsey (6). Substituting these data (for want of living cell values) into Equation 2 suggests that the increase in ΔH_v for the most weakly bound water (at 23% dry wt) would be about 360 cal/mole. The value for water in bulk is 10,440 cal/mole (16). If the last 1.5 mg of water evaporated from a conifer leaf (i.e. about 30% of the total water) had this higher ΔH_v, then a further $\frac{360}{18} \times 1.5 = 30$ mcal would be absorbed during its vapourization. This is measurable with the DSC cell of a Dupont 600 differential thermal analyser (DTA), as described below.

The DSC cell consists of a heating block supporting two identical aluminum calorimeter pans (each about 7 mm in diameter) in a convection-free enclosure (Fig. 1). The cell is also equipped with a system permitting vacuum maintenance over long periods of time. The thermal behaviour of the sample under study is recorded with respect to a

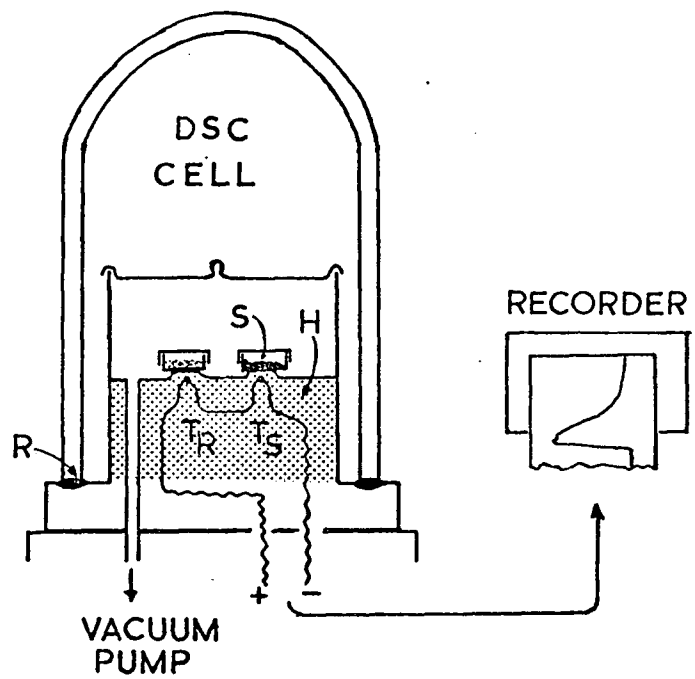

Fig. 1. Apparatus for vacuum differential scanning calorimetry. Heating block (H) supports samples (S) and reference calorimeters in a thermally uniform environment. Thermocouple series T_R and T_S senses the temperature depression in the sample as water is evaporated under vacuum. R is a rubber seal between glass cover and metal base.

thermally inactive reference sample of equal heat capacity. The temperature difference between the two calorimeters, ΔT, is measured by the sensitive thermocouples, T_R and T_S , in series, and forms the input to a strip chart recorder. The heat absorbed or evolved during a transition can then be calculated from the area under the exotherm if an empirical constant for the instrument is first determined, relating the known enthalpy change of a standard pure substance to its recorded exotherm area under the same operating conditions.

In the present study the block temperature was held constant by setting the instrument to "isothermal mode", and water was removed from a sample under vacuum. This avoids errors due to poorly matched thermal capacity of the reference sample, because the reference itself undergoes no appreciable temperature change. Heat of vapourization is given by:

$$\Delta H_v = \frac{mt\ \Delta T\ E'}{M_v} \qquad (3)$$

where M_v is the mass of water evaporated during the time, t(min), during which an endotherm is recorded, ΔT is the temperature depression of the sample calorimeter below the reference calorimeter, E' is the calibration constant (cal/°C-min), and m the molecular weight for water. In practice the endotherm is a rough curve (Fig. 2) in which ΔT varies with time. Hence ΔH_v is calculated from:

$$\Delta H_v = \frac{mAE}{M_v} \qquad (4)$$

where A is the area under the curve, and E is expressed directly as cal/cm^2 of endotherm area.

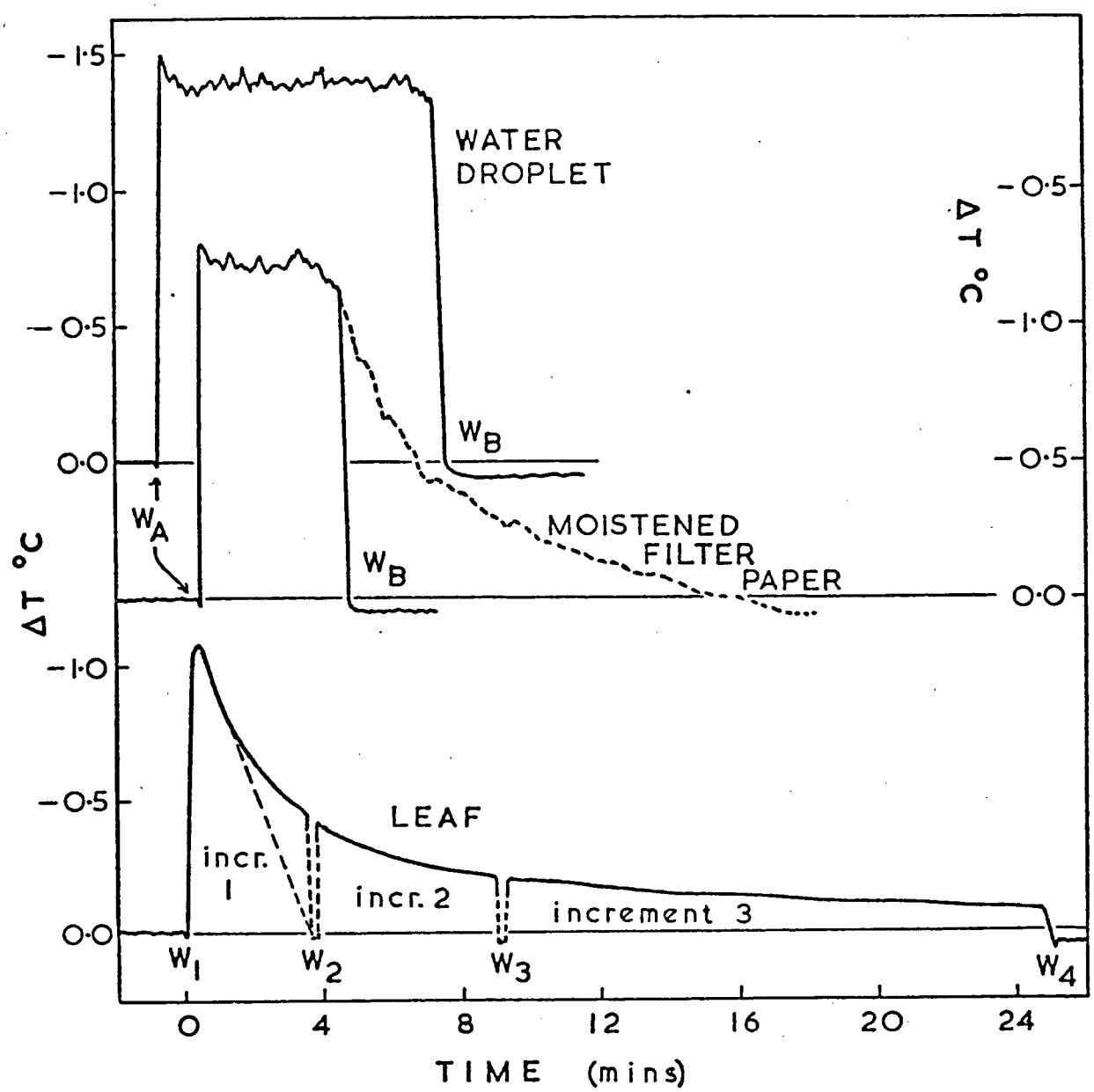

Fig. 2. Typical vaporization endotherms for water evaporated under reduced pressure at 30°C. ΔT is the temperature difference between sample and reference calorimeter pans. The rectangular part of the middle curve is assumed to be "free water." W_A and W_B are weights of samples used for calibration, immediately before and after evacuation. Leaf water (lower curve) was removed in 3 increments, with sample weights W_1 to W_4 being recorded before and after each. Position of W_2 was defined by intersection of a tangent with the baseline. W_3 was recorded when ΔT had decreased to half its value at the beginning of increment 2. W_4 was recorded when ΔT equaled 0.075°C with respect to the original baseline. In some runs W_2 was the only intermediate weighing.

Operating Procedure for Calorimetry

The following procedure was carried out at an isothermal temperature setting of 30°C. A leaf was placed, partly coiled up but undamaged, in a weighed (W_1) calorimeter pan. Pan and sample were reweighed (W_2, $\pm$ 0.2 mg) and quickly transferred to the DSC cell. Pressure in the cell was reduced within 10 sec to 25 mm Hg by opening the tap to a vacuum pump. The endotherm was recorded on an external time-base recorder at a speed of 2.54 cm/min. Attenuation of the recorder was adjusted to accommodate ΔT (usually 100 mV, i.e. 0.1°C/cm). The vacuum was released at an intermediate point in the endotherm, defined as shown in Figure 2, to include primarily the free water in the sample. The calorimeter plus sample were then reweighed (W_3) and returned to the DSC cell. A second intermediate weight (W_4) was obtained in some samples by a repetition of this procedure. The run was terminated when $\Delta T = 0.075°C$, below which the error in E becomes unduly large (see below), and a fifth weighing (W_5) made. An oven dry weight was recorded after 24 hr at 105°C. It was established that there was no measurable loss or gain of water from the air during the weighing period. Throughout each run the actual base temperature, T, was recorded ($\pm$0.05°C), with respect to an ice-water reference, on the DTA recorder so that corrections could subsequently be made for departures from the isothermal condition. Runs were conducted alternately on hardy and nonhardy leaves from the same plant. The leaves had been allowed to equilibrate at 100% humidity and 0°C overnight. This procedure was repeated for 10 needles

from each branch, for three seedlings, over a period of 10 days.

Determination of E

The calibration constant, E, was determined from Equation 4 by using a small droplet (3 to 5 mg) of water for the sample and taking the tabulated value of ΔH_v corresponding to base temperature, T (16). M_v was the difference between initial and final weights of the calorimeter plus sample (W_A - W_B in Fig. 2). A was determined ($\pm$ 2 mcal) by planimetry. For further determinations, water was evaporated from rolled-up pieces of filter paper to more realistically simulate the thermal and diffusive characteristics of leaves. In this case W_B was measured before the endotherm deviated from the rectangular form typical of free water.

The value of E was found to vary inversely with ΔT, because heat transfer between sample and block becomes less efficient when the rate of vapourization is low; a greater proportion of heat is dissipated. Therefore, a series of determinations of E was made corresponding to different rates of vapourization of water, the latter quantity being varied by small changes in vacuum pressure. (The density of air in the vacuum was assumed not to directly affect heat exchange significantly.) E was plotted as a function of ΔT (Fig. 3), and takes the form:

$$E = b_1 + \frac{b_2}{\Delta T} \qquad (5)$$

The constants b_1 and b_2 were determined by the method of least squares. The three determinations of E made with a droplet lie on the same curve

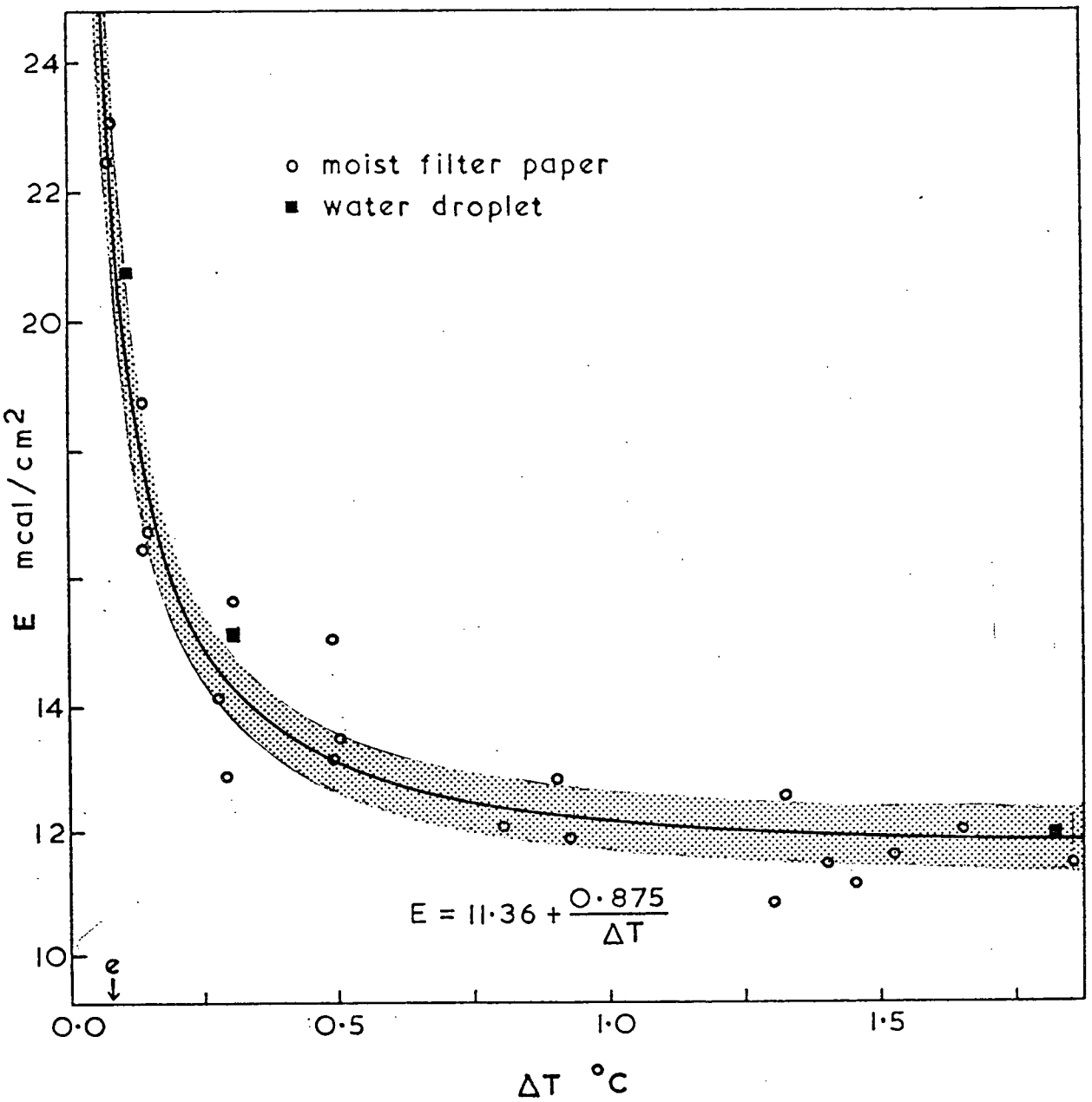

Fig. 3. Calibration curve for isothermal vacuum vaporization of water in the differential scanning calorimeter. E is the heat equivalent to 1 cm^2 of endotherm area (at recorder settings given in text). Shaded area represents 95% confidence limits on the regression line. Runs with leaf samples were terminated at e (ΔT = 0.075°C) to avoid excessive error in E.

as those made with moistened filter paper, indicating that errors due to the geometrical disposition of water in the calorimeter were negligible.

E is also subject to small variations between runs because some of the heat for vapourization is supplied by the sample itself (and is not recorded by the block thermocouple, T_S), and the sample mass varies between runs. However, even with a 25% increase in the sample's water weight (say, from 4 to 5 mg), and where $\Delta T = 1.5°C$, the additional heat contributed by the sample (i.e. the underestimate of $M_v \Delta H_v$) will be only about $1.5 \times (5 - 4) = 1.5$ mcal. This is close to errors in area measurement and weighing, and can therefore be ignored.

Computation of H_v for Leaf Water

The curved endotherms for leaf water (Fig. 2) were converted to digital form using a Gravicon Digitizer. This instrument automatically transfers the coordinates to punched cards as a pen is moved manually along the curve. ΔH_v was then computed from:

$$\Delta H_v = \frac{\Sigma (aEK)}{W_n - W_{n+1}} \qquad (6)$$

where a is the area under a very small segment ($\simeq$ 2 mm) of the curve, assumed rectangular, E is the calibration constant for the segment (calculated from Equation 5), K is the ratio of ΔH_v for pure water at 30°C (the isothermal setting) to that at T°C (16), and W is the weight of calorimeter plus sample at the start of evaporated increment, n (= 1, 2 or 3).

Dehydration over LiCl

Four groups of five needles were removed from a branch, allowed to equilibrate at 100% humidity and 0°C overnight, and weighed (± 0.05 mg). Groups were then suspended by fine wire over 2.10 molar, 3.50 M or 4.96 M LiCl or over pure water in sealed containers at 0°C. The volume of solution was large relative to that of tissue. The dehydrating energy of the solutions (ΔF_v from Equation 1) was equal to that of ice at -10°, -20° and -35°C respectively[1]. These four treatments were applied to the hardy and nonhardy branches of four seedlings.

The suspended samples were weighed at intervals until (after 1 to 3 months) there was no further measurable weight loss over a 3-day period. It was then assumed that the most weakly bound water in the tissue had a ΔF_v equal to that of the external solution. Oven dry weight was obtained (± 0.02 mg) after 24 hr at 105°C.

RESULTS

Heat of Vapourization

The present calorimetric data must be viewed with caution because the observed differences, although in the expected direction, are five to six times greater in magnitude than theoretically predicted. Between-plant and between-leaf variation are also much higher than expected.

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[1] Vapour pressures for ice, supercooled water and aqueous solutions were obtained from International Critical Tables (16), and for highly supercooled water were calculated from the relationships given by Olien (17).

The residual (between-leaf) variation was least in the seedling (No. 2) having relatively large needles, presumably because errors in E, a and W (Equation 6) were proportionately small.

The data from seedling No. 2 are given in Table I. These data indicate (a) that the heat of vapourization increases significantly ($p = 0.02$) between successive increments, that is as the proportion of water remaining in the tissue becomes less; and (b) that, for any one increment, the heat of vapourization is significantly higher in the hardy leaf. These trends in the data from individual needles are apparent only if the order in which needles were measured is also included as a source of variation. Thus it appears that significant changes of ΔH_v occurred over time due to drift in the instrument, to diurnal changes in the plants before sampling, or to changes in the needles themselves during storage.

The data from seedlings No. 1 and 3 represent only two increments per sample and, although more variable, support the principal observations above. The mean values are plotted with seedling No. 2 means in Figure 4. A multiple regression analysis of these curves (with independent variables "seedling", percent water, and hardiness) again shows both increment and hardiness factors to be significant at the 0.05 probability level. Further cause to question the validity of the data, however, is given by the marked deviation of ΔH_v for the first increment (which is presumed to be largely free water) from the standard value for free water (also shown in Fig. 4). Possible sources of error are discussed below.

Table I. Heats of vapourization of water from hardy (H) and nonhardy (NH) needles of a Douglas-fir seedling.

Leaf pair	Branch	HEAT OF VAPOURIZATION ΔH_v kcal/mole Increment 1	Increment 2	Increment 3	Water content % of fresh wt
1	H	11.25	11.79		58.1
	NH	9.42	11.59		63.1
2	H	9.14	10.40		59.4
	NH	9.20	10.54		63.8
3	H	8.85	9.79	10.44	58.9
	NH	8.28	8.60	10.67	63.0
4	H	9.94	10.36	10.07	58.9
	NH	8.36	8.85	11.00	62.8
5	H	9.83	8.57	11.30	59.2
	NH	9.86	10.30	10.62	65.1
6	H	11.60	13.07	22.47*	58.4
	NH	8.69	10.47	12.92	65.8
7	H	9.96	10.56	12.64	58.1
	NH	9179	9.40	10.87	64.8
8	H	10.63	10.46	11.34	56.6
	NH	9.61	9.13	10.13	64.3
9	H	8.63	10.06	11.15	58.2
	NH	5.90	8.63	9.85	64.8
10	H	7.87	9.62	11.28	57.3
	NH	7.79	6.07	8.57	64.6
Mean	H	9.77	10.31	12.58	58.3
	NH	8.69	8.93	10.58	64.2

Data are from seedling No. 2. Paired H and NH values are from immediately successive runs. Needles having deviant values (*) are omitted from Fig. 3. Values of the second increment for leaves 1 and 2 are also omitted from means of this table but are included in analyses of Fig. 4. Analysis of variance of ΔH_v for leaf pairs 3 through 8 shows both increment and hardiness factors significant at the 0.02 level.

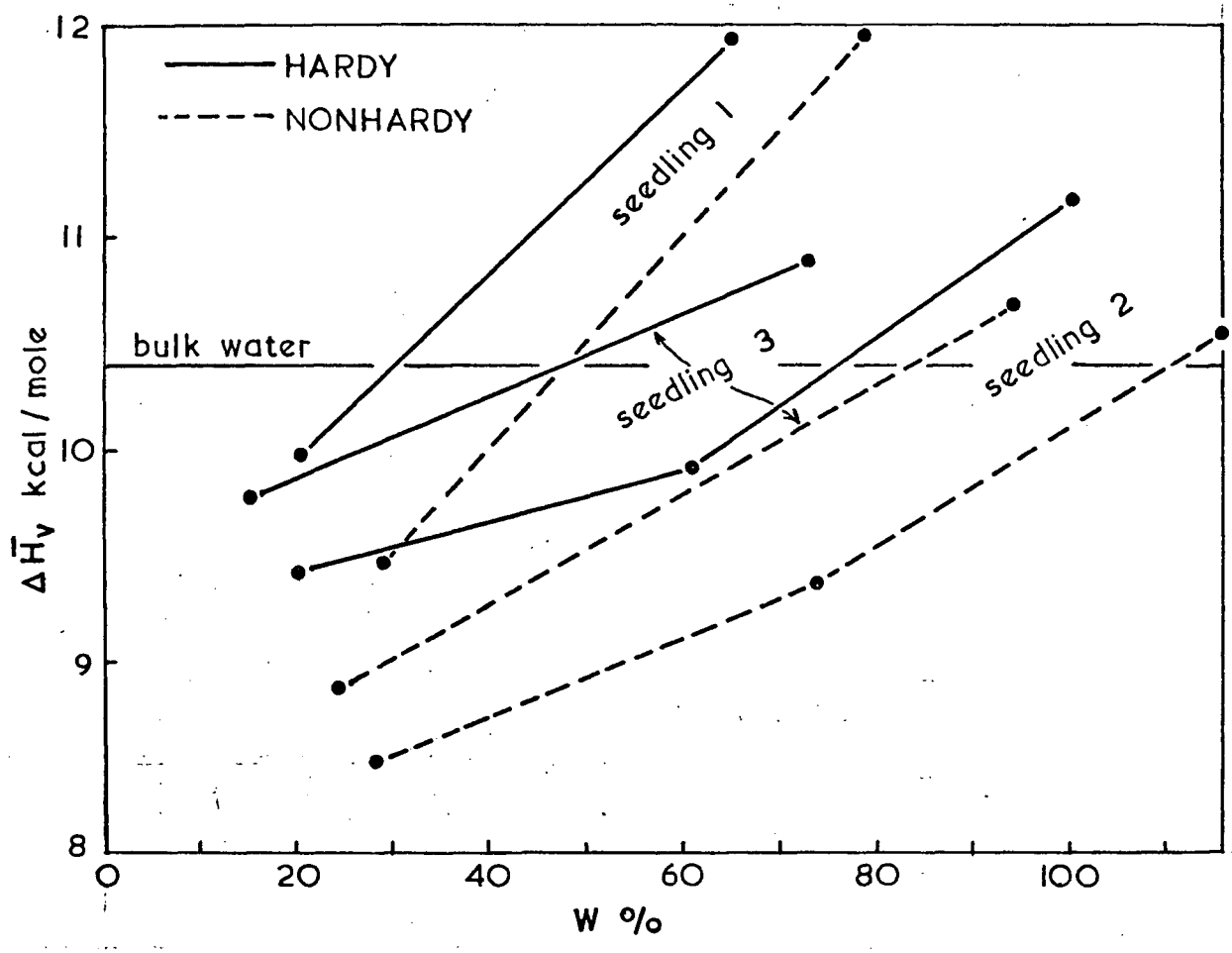

Fig. 4. Heats of vaporization for water removed under vacuum from excised needles. Each point is the mean of 10 needles from the same branch. W% is the average percentage of tissue water (dry weight basis) removed during evaporation, which was carried out in 2 or 3 stages. Variation in $\Delta \bar{H}_v$ due to hardiness is significant at the 5% probability level.

Dehydration over LiCl

The water retained by needles in equilibrium with LiCl isopeistic with ice at -10, -20 and -35°C is given in Table II. In all except one instance a greater proportion was retained in the hardy needles. Needles over pure water gained in weight, all hardy ones in this case equilibrating at a lower percentage water content.

These data, unlike those of the previous study, also reflect the osmotic component of retention, and no separate determination of this was attempted. However, the maximum value which the osmotic component can assume in hardy conifer leaves (3,12) is about 30 atm (=-13.6 cal/mole) compared with 10 atm (= - 4.36 cal/mole) in the nonhardy condition. It can be shown that this could account for only a part of the difference between hardy and nonhardy needles in Table II. For example, if a hardy leaf with a cell sap osmotic potential of -13.6 cal/mole loses 80% of its water while suspended over a LiCl solution of ΔF_v = -190 cal/mole, the osmotic potential in it becomes $-13.6 \times \frac{100}{80} = -68$ cal/mole. Therefore, the net dehydrating energy of the external solution, taking into account the cell solute, $\Delta F_v' = -190 - (-68) = -122$ cal/mole. If percent water retention is plotted against these adjusted values of ΔF_v, a new value of percent water, corresponding to -190 cal/mole can be obtained from the curve. This value represents a conservative estimate of the surface-bound water fraction at that dehydrating stress. Such estimates are given for each stress level in parentheses in Table II, and are significantly higher ($p = 0.01$) for hardy needles.

Table II. Water retention by needles at equilibrium with LiCl at 0°C

Seedling	Branch	Leaf water percent of dry wt at equilibrium with LiCl solution		
		2.10 M (-10°C)	3.50 M (-20°C)	4.96 M (-35°C)
3	H	75.3 (38.0)	75.3 (38.0)	35.2 (33.5)
	NH	72.4 (61.0)	39.1 (31.5)	26.8 (24.5)
4	H	105.6 (68.5)	53.3 (40.0)	35.4 (27.0)
	NH	55.4 (48.0)	38.2 (34.5)	25.1 (22.0)
5	H	91.7 (66.0)	61.2 (51.5)	46.7 (42.0)
	NH	94.8 (65.0)	54.5 (44.0)	39.5 (37.0)
6	H	93.5 (75.0)	69.0 (45.5)	36.6 (25.0)
	NH	69.5 (56.5)	39.1 (36.5)	34.7 (33.0)
Mean	H	91.5 (61.9)	55.8 (43.4)	38.5 (32.0)
	NH	73.0 (57.6)	42.7 (36.6)	31.5 (29.1)

M indicates molarity. Equivalent ice temperature is given. H = hardy (average killing temperature ≃ -35°C), NH = nonhardy (killing temperature ≃ -10°C). Values in parentheses are minimum estimates of the proportion retained nonosmotically (see text).

DISCUSSION

The data from both experiments support the hypothesis that non-osmotic binding of water contributes to a greater dehydration avoidance in hardy Douglas-fir needles. In making this statement the following possible sources of error have been evaluated.

(a) An error in the slope of the calorimeter calibration curve would lead to systematic differences in ΔH_v between increments (because both E and Increment No. are related to vapourization rate). The 95% confidence limits for the expected value of E are given in Figure 3. It can be shown that if the curve is moved anywhere within these limits the significant difference between increments (Table II) is not eliminated. Therefore, random error in the original calibration curve slope (not exceeding the 0.05 probability) is not a sufficient cause for the ΔH_v differences observed. Furthermore, vapourization rate was also somewhat greater in nonhardy needles, due to their greater content of water and probably also to greater stomatal apertures[2] (1), yet the nonhardy needles had higher, not lower, values for ΔH_v. It must be assumed that the curve has some error (perhaps associated with the necessarily different type of sample used in calibration) because free water and Increment 1 ΔH_v values do not agree. But this is a "level" rather than a "slope" error, and would not affect relative values of ΔH_v among treatments.

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[2] Van den Driessche, R. (1972), unpublished data.

(b) The presence of volatile oils will affect the results of both calorimetric and dehydration studies. Parker (18) recognized this difficulty in applying Levitt's method for bound water (10) to eastern red cedar leaves. In pines, the oils constitute up to 0.5% of the needle fresh weight (14), and have an average heat of vapourization[3] of about 80 cal/gm. Assuming the heats of vapourization of water and oil to be additive in mixture, the error can be calculated from

$$\Delta H_w = \frac{\Delta H_v W_v + \Delta H_o W_o}{W_w} \qquad (7)$$

where ΔH_o is the heat of vapourization for oil, ΔH_w the unknown value for water and ΔH_v the experimentally determined value for the oil-water mixture. W_o W_w and W_v are the corresponding weights. This error turns out to be small, so that in an average case ΔH_v for the first increment (assuming this to contain nearly all the oil) underestimates the true value for tissue water by only 1.7%. This correction is indicated in Figure 4 and would not affect the present conclusions unless nonhardy Douglas-fir needles contained 3 or 4 times as much oil as found in other conifers, while hardy needles contained little or no oil at all. It can also be seen that the amount of 1.25% of the dry weight, accounted for by volatile oils, is only a small part of the difference observed between hardy and nonhardy retention percentages in Table II.

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[3] This was calculated from tabulated vapour pressure/tempearture relationships (30) for several monoterpenes typically found in pine oil (14), using the Clausius-Clapeyron equation.

(c) Nonhardy needles contained up to 10% more water. If this additional 10% were all free water (the amount of "structured" water remaining constant), then there would be a decrease in the average ΔH_v of nonhardy needles. Arithmetic calculation shows that this decrease would be only about 1%, whereas observed differences between hardy and nonhardy needles averaged 5%.

It may be concluded that tissue water is at a lower (more negative) potential in frost-hardy Douglas-fir needles, thereby allowing these to more effectively avoid dehydration stress. While osmotic lowering almost certainly plays a role (11), there is an additional lowering presumably caused by the proximity of macromolecular surfaces. Cytological changes which lead to an increase in internal surfaces during hardening have been described by Pomeroy and Siminovitch (19).

The magnitude of this dehydration avoidance, due to the combination of osmotic and colloidal forces can be roughly estimated from Figure 5. This shows that the water content of hardy needles over a solution isopeistic with their killing temperature (-35°C) averages 38% of the dry weight. Nonhardy needles, however, have attained this critical water content at -23°C. Therefore, there has been a 12°C gain in hardiness as a result of dehydration avoidance by the hardy leaf. This assumes that dehydration by intercellular ice over a period of hours and over LiCl for several months are equally injurious. In fact, ice may cause somewhat more injury (17), or less injury (22) than isopeistic desiccation. In the present case the prolonged desiccation caused more

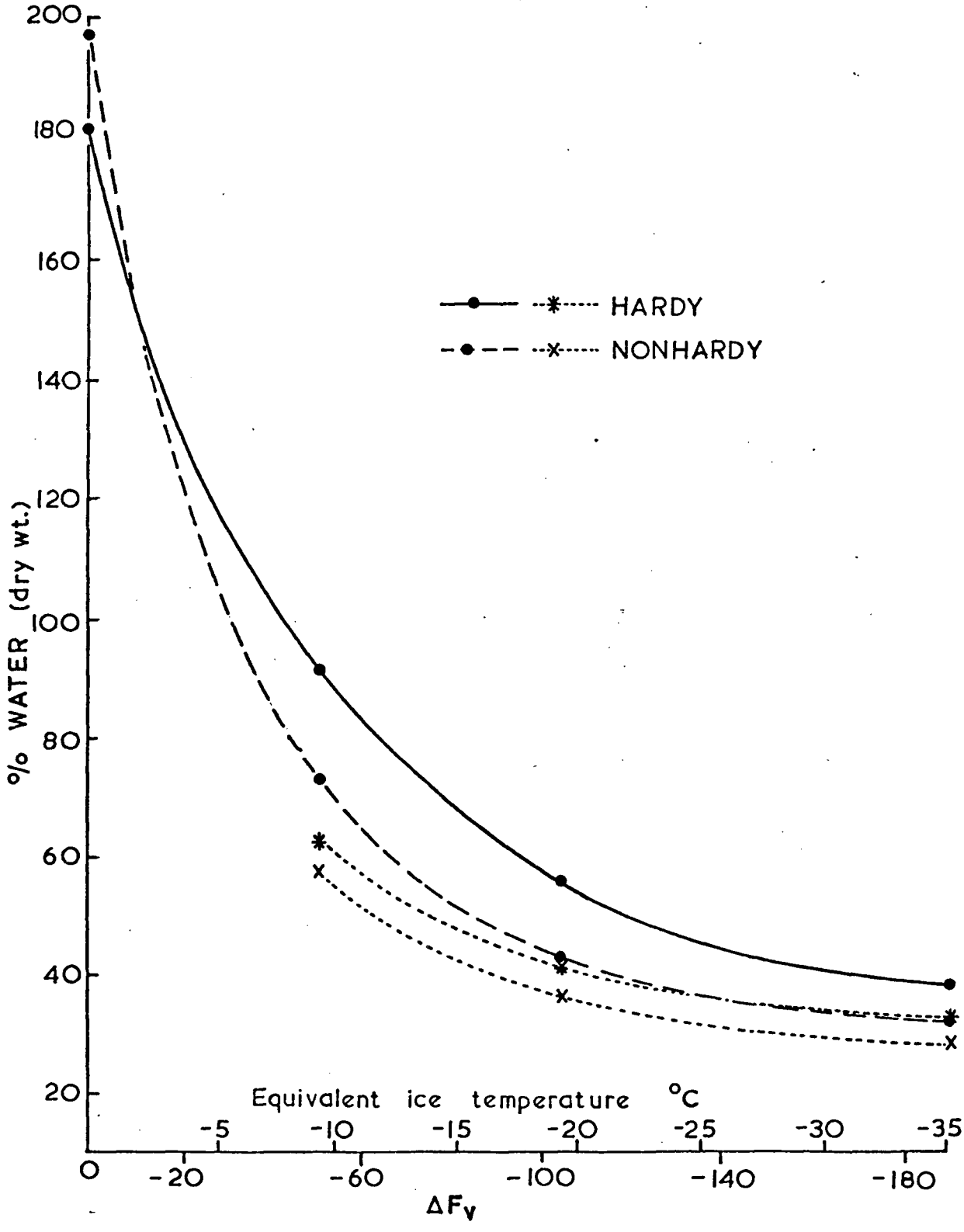

Fig. 5. Water contents of needles at equilibrium with LiCl solutions. Each point is the mean of 5-needle samples from the hardy or nonhardy branches of four seedlings. Lower pair of curves shows the effect of making a generous allowance for osmotic potential differences between hardy and nonhardy needles (see text). In both cases the retention curves differ significantly at the 5% probability level.

injury than freezing (data are not shown), and it is probable that the retention curves show smaller differences between hardy and nonhardy samples due to a gradual breakdown of physical structure in the tissue after excision. If so, 12° is a conservative estimate of the avoidance component. The contribution of surface binding to the total avoidance, based on the conservative estimates in Table II (of which means are plotted in Fig. 5), is about 9 centigrade degrees. These non-osmotic retention curves are also in agreement with the idea reviewed earlier, and suggested by the calorimetric data, that surfaces influence a relatively large mass of water with a wide range of energies rather than a small fraction at a discrete energy level.

The remainder of the difference between hardy and nonhardy needles on a plant -- about 13 centigrade degrees -- must be explained by some mechanism of dehydration tolerance, for which several theories have been proposed (11,31). Further study is needed to determine whether avoidance and tolerance adaptations are associated with specific "stages" of cold acclimation (27,31) and dehardening (28).

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