AN ABSTRACT OF THE THESIS OF

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Title: FREEZING RESISTANCE OF TISSUES IN THE TWIG OF DOUGLAS-FIR (PSEUDOTSUGA MENZIESII (MIRB.) FRANCO)

Abstract approved: _____
Dr. Richard K. Hermann

The relative freezing resistance of tissues in the stem, foliage and buds of terminal twigs from Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) was investigated at about ten-day intervals from summer until the following spring. Tissues from growing twigs collected before development of dormancy had no freezing resistance and were killed immediately after subcooling. Tissues at the base of inner bud scales and in the pith of stems at the base of buds were among the last to harden in autumn. Pith parenchyma and pith rays of the interfascicular region of the bud trace and the transfusion tissue in needles were most susceptible to injury after development of freezing resistance. Abaxial mesophyll was more susceptible to injury than adaxial mesophyll of hardened needles. The cortex was more resistant than other tissues during development of freezing resistance but parenchyma of the pith lost freezing resistance more slowly and was more resistant after growth processes resumed in the spring. The phloem-cambium region of the stem lost freezing resistance earlier and became more susceptible to injury than other tissues

of the twig after midwinter. Survival of the vegetative apex depended on the ability of its water to subcool. Heat of fusion of water on freezing produced a sharp exotherm that was a precise index of the temperature at death and freezing resistance of the vegetative apex.

Prolonged subfreezing temperatures after development of freezing resistance in late autumn increased hardiness of twigs beyond that induced by the natural environment. Both formation of ice in the pith of stems enclosed by coriaceous bud scales, and artificial dehydration reduced water content and promoted subcooling and freezing resistance of vegetative apices. Moisture content was reduced from 200 to 70 percent on a dry weight basis after three days at -9° C in early February and freezing resistance of apices increased from -16° C to -20° C when cooled at 1° C per minute. These findings suggested that diffusion of water from the protoplasm to sites of extracellular ice, and not low temperature, was the direct cause of hardening at subfreezing temperatures.

The basic freezing curve for Douglas-fir is characteristic of plant tissues in general but was modified by exotherms associated with freezing and death of vegetative apices from November until April. A decline in length of the plateau after the second subcooling temperature during hardening, and increase in length of the plateau during loss of hardiness reflected probable seasonal change in quantity of intracellular water. Unhardened tissues survived freezing of extracellular water but were injured before intracellular water was completely frozen. Hardened tissues, on the other hand,

were not injured until after most of the intracellular water was frozen and the cooling rate of the stem returned to normal.

Observations suggested a definite relationship between time of acquisition and loss of freezing resistance and number of days to bud burst after March 31. Trees which developed freezing resistance earliest in fall broke buds earliest in spring. Trees which broke buds latest in spring were also those which were the last to deharden. Ready availability of potassium appears to enhance early development and loss of freezing resistance. Trees with the highest content of potassium in the current season's foliage burst buds earliest and those with the lowest content of potassium were the last to burst buds. Night temperatures of 2° C delayed but did not prevent loss of hardiness when day temperatures were 25° C in March. However, continuous temperature of 2° C failed to reharden twigs that had lost freezing resistance at 25° C. Injury to all tissues, except the vegetative apex, declined at 25° C in March when moisture stress exceeded a critical level. These observations suggest that environmental conditions unfavorable for growth enable Douglas-fir to maintain resistance to injury from spring frosts.

Freezing Resistance of Tissues in the Twig of Douglas-Fir *Pseudotsuga Menziesii* (Mirb.) Franco)

by

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FREEZING RESISTANCE OF TISSUES IN THE TWIG OF DOUGLAS-FIR (PSEUDOTSUGA MENZIESII (MIRB.) FRANCO)

INTRODUCTION

Injury to forest trees and other plants from subfreezing temperatures has been the subject of extensive research for over a century. Much of the early work about freezing resistance of plants is reviewed by Maximov (1929), Belehradek (1935), Harvey (1936), Luyet and Gehenio (1940), Tumanov (1940), Levitt (1941 and 1956), Chandler (1954), Dexter (1956), Vasil'yev (1961) and Parker (1963). Survival of plants in subfreezing climates depends on both the ability and timing for development and loss of freezing resistance. Development of freezing resistance begins usually in midsummer and continues gradually as tissues differentiate and mature until early autumn when hardening accelerates. Maximal freezing resistance is attained in late autumn and early winter and dehardening begins slowly in midwinter. Loss of freezing resistance accelerates with the appearance of warm weather and initiation of growth processes in late winter or early spring. All freezing resistance is lost when growth becomes rapid in late spring.

This annual cycle of development and loss of freezing resistance is governed by physiological processes that have evolved through plant and environment interaction. These processes are probably initiated by the same environmental factors that reduce growth and initiate dormancy. Numerous studies have shown that temperatures below about 0° C, short photoperiods, diminishing soil moisture and

availability of certain inorganic nutrients are major environmental factors that induce plants to develop freezing tolerance. Availability of potassium is particularly important for development of freezing resistance in forest trees and nursery stock (Benzian, 1966 and Leaf, 1968). High levels of nitrogen, on the other hand, prolong growth and delay hardening.

Seasonal variation in freezing resistance has been investigated for relatively few plant species although numerous studies have been conducted to determine the causes of injury from cold and how plants develop resistance to freezing. Most investigations of seasonal changes in freezing resistance have been conducted on plants from continental climates where winters are severe, early and late frosts are frequent, and freezing injury is common. By contrast few detailed studies have been made on the freezing resistance of species native to oceanic climates where winters are mild.

Furthermore, investigators have found deciduous species more attractive for study of freezing resistance than coniferous species for several reasons. Conifers develop resistance more slowly and are less hardy (Parker, 1963). The ability of cuttings from some deciduous species to root and put forth buds makes tests of viability easy after freezing. Also, conifers have high polyphenolic contents that form crosslinks with proteins and other macromolecules to inhibit activity of enzymes and organelles in cell free preparations.

Survival of Douglas-fir in new climates depends on its ultimate freezing tolerance and biological timing for development and loss of resistance to freezing injury. For this reason, freezing resistance

of Douglas-fir has been investigated occasionally in conjunction with provenance studies in Europe. Early studies indicated that Douglas-fir was particularly susceptible to autumn frosts (Abele, 1909; Dannekat, 1928; Manshard, 1928). Abele (1909) observed that trees two to six years old were prone to injury from early frosts when environmental factors such as excessive precipitation, high nitrogen fertility, heavy clay soils and south or west exposures prolonged growth and delayed hardening.

In North America, Clements (1938) observed that the foliage of Douglas-fir near Pullman, Washington, was severely injured at -15° C in November but survived -30° C in January. Young needles were slightly injured at -15° C in early April when seasonal moisture contents were minimum. More recently, Edgren (1970) reported that seedlings of the coast variety (P. menziesii var. menziesii) were severely injured by early frosts at the Wind River Nursery near Carson, Washington. Seedlings from high elevations and east of the Cascades were more resistant than seedlings from low elevations and the Coast Range. Severely injured seedlings appeared to have been growing faster than uninjured seedlings from the same sources before freezing. Injured seedlings that were left undisturbed in the nursery beds for seven months before transplanting grew faster and were larger than uninjured seedlings after two years. In Germany, Kappen (1968 and 1969) studied the seasonal change in freezing resistance of the foliage of Douglas-fir from ten provenances and found that the interior variety (P. menziesii var. glauca) hardened earlier and developed greater freezing resistance than the coast variety.

Provenances from high elevations developed greater freezing resistances than provenances from low elevations. However, van den Driessche (1970) found no differences in the seasonal change in freezing resistance of coastal Douglas-fir seedlings from high, medium and low altitude provenances grown at three nurseries in British Columbia.

Some major environmental effects on the freezing resistance of Douglas-fir have been studied recently. McGuire and Flint (1962) observed that several conifers including Douglas-fir failed to harden at temperatures from 0° to 5° C in the absence of light. Börtitz and Weise (1963) found that respiration in continuous darkness at -2.5° C depleted sucrose reserves that may have been essential for high freezing resistance of hardened Douglas-fir seedlings. However, intensive respiration during recovery after short periods of freezing was a characteristic of the most metabolically active and hardy type of Douglas-fir found in the same provenances with a less hardy type (Weise and Polster, 1962). Scheumann and Börtitz (1965) separated the hardening phase of Douglas-fir and Norway spruce (Picea abies (L.) Karst.) into periods of response to temperatures above and below 0° C. Light in the form of 12-hour photoperiods was essential for hardening above 0° C and maintaining hardiness below 0° C. Douglas-fir remained hardier longer at 15° C if light was present during both periods of hardening. They concluded that photoperiod was more important than low temperatures in triggering the hardening process in Douglas-fir. Van den Driessche (1969a) also found that short photoperiods increased hardiness more than

long photoperiods when day temperature was 13° C and night temperature was 7.5° C. However, long photoperiods did not prevent development of freezing resistance and continuous light at low intensities caused more hardening than eight-hour light periods after nine weeks at 2.5° C. Seedlings in darkness did not harden at 2.5° C.

The general objective of this thesis was to investigate seasonal changes in the freezing resistance of major tissues in the twig of coastal Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco). Specific objectives were:

1. To determine if Douglas-fir is capable of developing additional freezing resistance in response to low temperatures and dehydration after hardening in its natural environment;
2. To relate freezing resistance of the vegetative apex with moisture content and arrive at a mechanism of hardening at subfreezing temperatures;
3. To investigate seasonal change in the freezing curve for tissues of the stem under the terminal apex and relate injury to time, temperature and position of the freezing curve after initial formation of ice to gain information about the site and cause of freezing injury;
4. To determine the effects of low temperature, dehydration and light on loss of hardiness and rehardening of tissues after twigs are exposed to warm temperatures in March;
5. To determine the effect of potassium nutrition on development and loss of freezing resistance.

METHODS

Sensitivity of Douglas-fir to freezing was determined by freezing twigs in a chamber constructed for that purpose. The freezing chamber contained an ethylene glycol bath and was part of an apparatus designed by Wessel and Hermann (1969) to control rates of temperature change and to hold temperatures at a desired freezing level.

Terminal twigs, 5 to 8 cm in length, were collected from field-grown Douglas-fir trees in all experiments. The twigs were immediately brought to the laboratory and frozen in 25 x 100 mm test tubes. Temperature of the tissues in two excised twigs chosen at random from each sample was measured with a 30-gauge (0.01 inches in diameter) copper-constantan thermocouple inserted through the cortex and vascular tissue into the pith of the stem directly under the terminal bud. Temperature of each thermocouple was recorded continuously during each freezing test on a Sargent dual channel recording potentiometer. Chart speeds of 3 and 30 inches per hour were used at slow (3° C per hour) and rapid (1° C per minute) rates of freezing, respectively. The span was adjusted to one millivolt full scale to provide a temperature range from 0° to -26.9° C.

The twigs were allowed to stand either at room temperature or in continuous light at 2° C for 24 hours with their severed ends in water after freezing. Light intensity during all recovery and hardening treatments was about 20 foot-candles. Injury was evaluated 10 to 15 days after freezing according to the extent of browning

that occurred in the major tissues of the stem, bud and needles. Injury to the cortex, phloem-cambium and pith regions of the stem was evaluated in transverse section under a Bausch and Lomb binocular microscope at magnifications of 15x and 36x. These transverse sections were of uniform diameter and were taken between 0.5 and 1.5 cm below the base of the terminal bud. The mesophyll and tissues inside the endodermis were examined in longitudinal sections of needles from the same region of the stem as the transverse stem sections. Vegetative apices, scales of terminal and lateral buds and tissues of the stem enclosed by coriaceous bud scales were examined in radial sections. Freehand sections of the above tissues were examined with a Zeiss microscope at a magnification of 90x when initial stages of injury were in question. All frozen tissues were compared with unfrozen tissues of control twigs from the same tree. Browning developed more quickly in tissues injured by freezing than in uninjured tissues when excised sections were exposed to air.

The extent of injury from freezing could not be determined by external appearance alone after tissues matured in late summer. For this reason freezing injury was determined by evaluating the degree of browning in tissues 10 to 15 days after the twig was frozen. The coloration in each tissue from a frozen (test) twig was compared with the same tissue from an unfrozen (control) twig and rated according to the following scale of injury.

Damage Index

1.00 No apparent injury

The frozen tissues have no discoloration when compared with the same tissue from an unfrozen twig. Cells of the cortex and pith are green, the phloem-cambium is dark and cell walls of mature tracheids are white. Central parenchyma of the pith in stems may be dead (brown) from natural causes. Pith parenchyma under the crown of vegetative apices are white after these cells mature.

1.25, 1.50 Initial injury

A light brown discoloration is apparent in the cortex, phloem-cambium or pith tissues, for example. Pith tissue may have a dark, water-soaked appearance that becomes faintly brown after it is exposed to air for several minutes. A brown mottled appearance in the pith, spongy mesophyll, xylem, and inside the endodermis of the needle, or a few brown streaks in the endodermis of the needle and xylem of the bud trace and stem are examples of initial injury. Initial injury in fully hardened tissues results from disruption of pith parenchyma adjacent to the primary xylem, parenchyma of the interfascicular region, xylem ray and transfusion tissues. Albuminous cells of transfusion tissue may also be susceptible to initial injury. Injured cells of the phloem-cambium region as well as the

pith may cause discoloration of the xylem. Tissues with a mild water-soaked appearance are considered uninjured unless they brown quickly in air. Initial injury is apparent when 1 to 15 percent of all cells are destroyed. Injury is slight and tissues will recover without adverse effects.

1.75, 2.00, 2.25 Advanced injury

Brown color in the injured tissue is prominent. Injury may also be distributed throughout the tissue or confined to one region. For example, all tissues of needle tips may be killed while the central and basal portions are uninjured or a brown discoloration in the vascular region may extend throughout the entire needle. The number of cells destroyed may range from about 15 to 85 percent. Tissues with injury between 1.50 and 2.00 will probably recover but some mortality and deformity is expected. Recovery of tissues with injury between 2.00 and 2.50 is unlikely. Injury is expected to reduce and cause abnormal growth if the tissue survives.

2.50 Excessive injury

Brown color in the injured tissue is extensive. The tissue may be disrupted from massive ice formation but a few

cells remain viable. Tissues with excessive injury will not recover.

3.00 Dead

Tissues contain no living cells. Unhardened tissue is frequently disrupted from massive ice formation.

Injury was abrupt, extensive, and easily defined after initial formation of ice in unhardened tissues. However, after tissues developed resistance, injury increased gradually with decreasing temperature and time of exposure. For this reason stages of injury intermediate to the midpoint values were frequently apparent after the trees developed hardiness. These stages were evaluated as 1.25, 1.75, 2.25 and 2.75. Examples of uninjured (not frozen), injured and completely destroyed tissues of stems are shown in Figures 1, 2 and 3.

The cause of browning that occurs after frost injury is unknown. However, hydrogen bonds between phenols and proteins and oxidation of polyphenolic compounds to quinones followed by covalent condensation with reactive groups of proteins, are sources of browning during isolation of plant enzymes and organelles (Swain, 1965). Loomis and Battaile (1966) reviewed ways that phenolic compounds react with proteins and compounds related to proteins. Krasavtsev (1962) mentioned that browning develops after cell death and is associated with destruction of protoplasmic structures. Initial injury apparently occurs during freezing when certain polyphenols and their glucosides react with substances containing amino acids to produce

Figure 1. Transverse section of an unfrozen stem collected on January 29. Note absence of browning that was characteristic of injured stems. 40x.

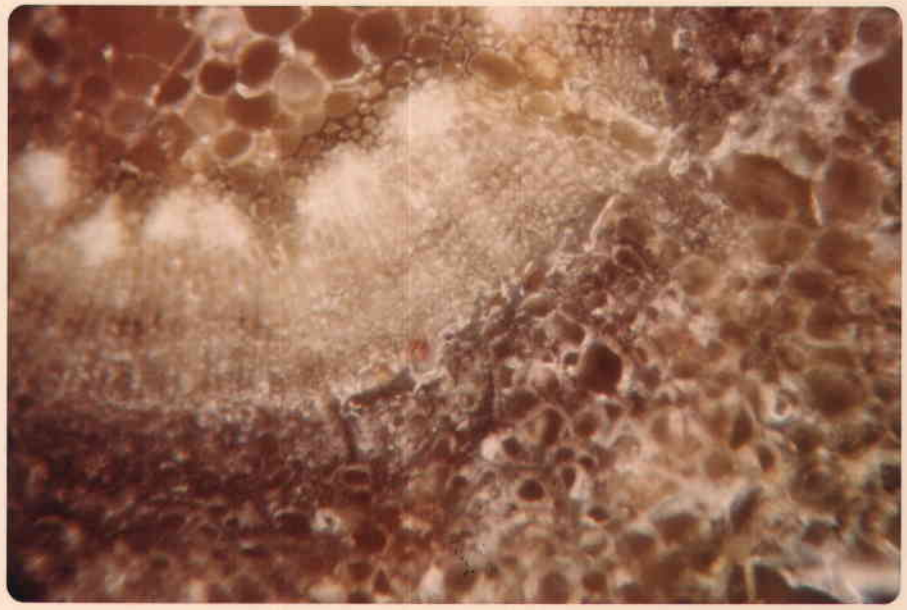

Figure 2. Transverse section of a stem frozen to -22° C at 3° C per hour on January 29. Damage index: pith, 1.50; xylem, 1.50; phloem-cambium, 2.50; cortex, 1.00. Note the wide dark band and extensive injury in the phloem-cambium region. 40x.

Figure 3. Transverse section of a stem frozen to -25° C at 20° C per hour on January 30. Damage index: all tissues, 3.00. Note the white crystalline structure of secondary walls of tracheids after the section has dried. 80x.

a greenish-yellow fluorescence that is stable while oxidative processes are inhibited at subfreezing temperatures.

In my experiments, evaluation of injury on the basis of browning after freezing provided a satisfactory method of estimating maximal resistance to freezing and for determining relative differences in freezing resistance between tissues of the stem, needle and buds. However, a delay of one week or longer after freezing was necessary for formation of substances that cause browning. Degree of browning following injury was particularly intense in parenchyma of pith and transfusion tissues which contain high polyphenolic contents. Browning products diffused from severely injured into adjacent tissues and sometimes gave a wrong impression of the extent of primary injury from freezing. Evaluation of injury was complicated further by the fact that it could not be estimated more accurately than to the points of the damage index scale. For this reason, injury could not be measured as a continuous random variable, and frequency distributions of samples approach but do not form normal probability distributions. The major statistical technique used to determine the effect of treatment on injury was analysis of variance, however. Sources of variation were determined for treatment, trees, and treatment x tree interactions. Regressions of independent variables such as temperature, time, date of bud burst, and percent potassium content against injury were used for some of my experiments.

SEASONAL CHANGES IN FREEZING RESISTANCE

One of the earliest studies of seasonal changes in the relative resistance of major tissues in the twigs of a woody plant was made by Chandler (1913). He observed that the cortex, cambium and young sapwood of unhardened apple twigs were more susceptible to injury than the pith during the summer but more resistant to injury after growth ceased in the autumn. Although tissues of the xylem and cortex browned extensively after freezing, hardened twigs survived because the cambium was not injured. Cortical parenchyma also hardened more quickly under natural and artificial conditions than cells of pith periclinal tissue in willow and poplar (Sakai, 1966) and xylem tissue of grape was more hardy than phloem and ring parenchyma tissue of the node in midwinter (Pogosyan and Sarkisova, 1967).

In more recent studies concerning conifers Scheumann (1962) observed that needles of larch were more resistant than cambium and bark tissues in the spring and Day and Barrett (1963) reported that needles of Douglas-fir and several other conifers were injured mostly in winter but buds and the previous year's growth were injured more frequently in the spring. Twigs of todo fir (Abies sachalinensis Fr. Schm.) and white spruce (Picea glauca Voss.) were less resistant than buds during the autumn (Takatoi, Watanabe and Kamada, 1966). However, the twigs became more resistant than the buds after mid-November.

One of my objectives was to determine what tissues of Douglas-fir are most susceptible to freezing injury because hardiness of the twig

as a whole depends on the susceptibility of all tissues and information of this nature is meager for conifers in general and for Douglas-fir in particular.

In addition, I investigated the effect of potassium deficiency on seasonal changes in freezing resistance of major tissues of Douglas-fir in conjunction with this part of the study. Potassium nutrition has been shown to play a role in frost hardening processes of trees and nursery stock (Leaf, 1968) and potassium fertilizers applied to seed beds of Sitka spruce (Picea sitchensis (Bong. Carr.) are known to decrease frost injury (Benzian, 1966). Potassium is a univalent cation that activates more than 40 different enzyme complexes (Evans and Sorger, 1966) of important biochemical processes including glycolysis, starch synthesis, oxidation and photophosphorylation, respiration, amino acid metabolism, protein synthesis and nucleic acid metabolism. Development of maximum freezing resistance in woody plants is associated with an increase in total protoplasm (Siminovitch, 1963; Siminovitch et al., 1968; Pomeroy, Siminovitch and Wightman, 1970) that undoubtedly depends on activity of these processes and adequate potassium nutrition. Potassium ions in the diffusate from stems of hardened Douglas-fir are less than in the diffusate from unhardened stems (van den Driessche, 1969a) and high levels of potassium increase the impedance and freezing resistance of alfalfa (Hayden et al., 1969). These findings indicate that potassium accumulates in the intracellular rather than extracellular space of plant tissues and that freezing resistance is probably enhanced by synthesis of amino acids, proteins and carbohydrates.

Procedure

Cuttings from ten-year-old Douglas-fir trees under intensive management for Christmas tree production were collected for freezing tests every 10 to 14 days, and at shorter intervals when substantial changes in freezing resistance were expected. Initially, 12 trees each were selected at random in potassium-deficient and nondeficient areas in a plantation that is located at 450 feet above sea level, ten miles southwest of Corvallis in Section 8, T13S, R6E, Willamette Meridian. Aspect of the potassium-deficient area is east and the nondeficient area is south. Trees growing on the nondeficient area were from a local seed source while those growing in the potassium-deficient area were from an unknown seed source, probably from somewhere in the Willamette Valley.

To obtain more information on effect of potassium, 18 additional trees were selected on the deficient area. Nine trees were selected for their deficiency symptoms while the remaining nine trees were selected for their vigor to obtain samples from trees representing a range of potassium contents. The current season's foliage of six trees from each area was collected in July and October for analysis of its potassium content. The current season's foliage from all trees was sampled in January and March. The foliage was dried at 70° C for three days, ground to a 40 mesh size and 0.4 g samples were prepared for potassium analysis by a modification of the wet ash technique from the Annual Official Methods of Analysis of the Association of Official Agricultural Chemists. Potassium content

of each sample was determined with a Beckman DU flame spectrophotometer, Model 2400.

Date of flushing, as a measure of the growth response, was recorded as the number of days after March 31 until emergence of the first new needles on each tree. Date on which new growth appeared on the north side of the sample trees and percent of trees fully flushed at the final freezing test was also recorded. Date of bud burst is a crude estimation of onset of growth processes, however, because vegetative apices begin to enlarge several weeks before bud burst, and succinic dehydrogenase activity in vegetative apices becomes apparent about two months before bud burst (Owens, 1968).

Eight trees in different stages of flushing were selected from the potassium-deficient area on May 2 to determine if resistance to injury from freezing varies between twigs from the same tree. Three twigs on both the north and south sides of each tree were frozen to -6° C and difference in injury between aspect and tree was determined by analysis of variance. Development of buds on each aspect of the tree was estimated according to the following scale:

0 - No evidence of bud enlargement.

2 - Buds are swollen but bud scales are not separated.

4 - Tips of the buds are white and bud scales have separated.

6 - Bud enlargement is pronounced and bud scales are definitely separated.

8 - Buds are on the verge of breaking and bud scales are widely separated.

9 - Needles have emerged from the bud.

The effect of bud enlargement on injury to tissues of the twig was determined by simple regression analysis.

After twigs were cut from sample trees they were immediately placed in 25 x 100 mm test tubes in an ice chest at 8° to 10° C and returned to the laboratory for freezing within one-and-one-half hours. The twigs were cooled and warmed at 3° C per hour and held at a predetermined test temperature for two hours. Test temperatures during hardening were selected to produce severe injury and samples were frozen at this temperature in subsequent trials until injury became negligible. A new test temperature 4° to 6° C lower was then selected. This process was repeated until the twigs reached maximum hardiness. The procedure was reversed after trees began to lose hardiness in midwinter and test temperatures were raised at intervals of 4° C.

The twigs were removed from the ethylene glycol bath after completion of the freezing test and placed in continuous light for 24 hours at 2° C for recovery. Injury was photographed periodically using an Asahi Pentax camera with a 50 mm lens, a +2 supplemental lens, a +3 supplemental lens and a 65 mm extension tube to provide 1:2 magnification on 35 mm film. Freehand sections showing various stages of injury during the period of maximum resistance were photographed through a Zeiss microscope with interchangeable lenses at magnifications from about 40x to 240x.

Terminology in describing various tissues of the twig follows

Esau (1967) and Owens (1968). For definition of tissues, see schematic drawing of anatomy of twig (Figure 4).

Difference in Injury Between Tissues

Twigs collected during July were succulent and immature although all bud scales were initiated. Freezing trials during this period caused instant death from formation of ice in tissues of the stem and injured twigs were clearly discernible from uninjured twigs (Table I). Needles often remained viable after the stem was killed, however.

Injury to twigs frozen during August was no longer clearcut (Table I). Twigs lost their succulent appearance and needles as well as stems were partially injured in August. Current season's growth on the nondeficient trees appeared more mature than the current season's growth on the potassium-deficient trees. Tissues of the twig apparently developed little or no resistance to injury from ice formation but quantity of ice that formed was less or nucleation of ice was retarded because moisture contents were less. Ice may also have been confined to tissues in which it was initially formed because lowered moisture contents and dehydration of surrounding cells, as freezable water diffused to sites of ice formation, inhibited migration of ice into adjacent tissues. The increase in number of partially injured stems and needles and reduction in number of stems that were completely killed reflected these changes.

Although injury at the same test temperatures decreased slowly during September, the first substantial increase in hardiness did

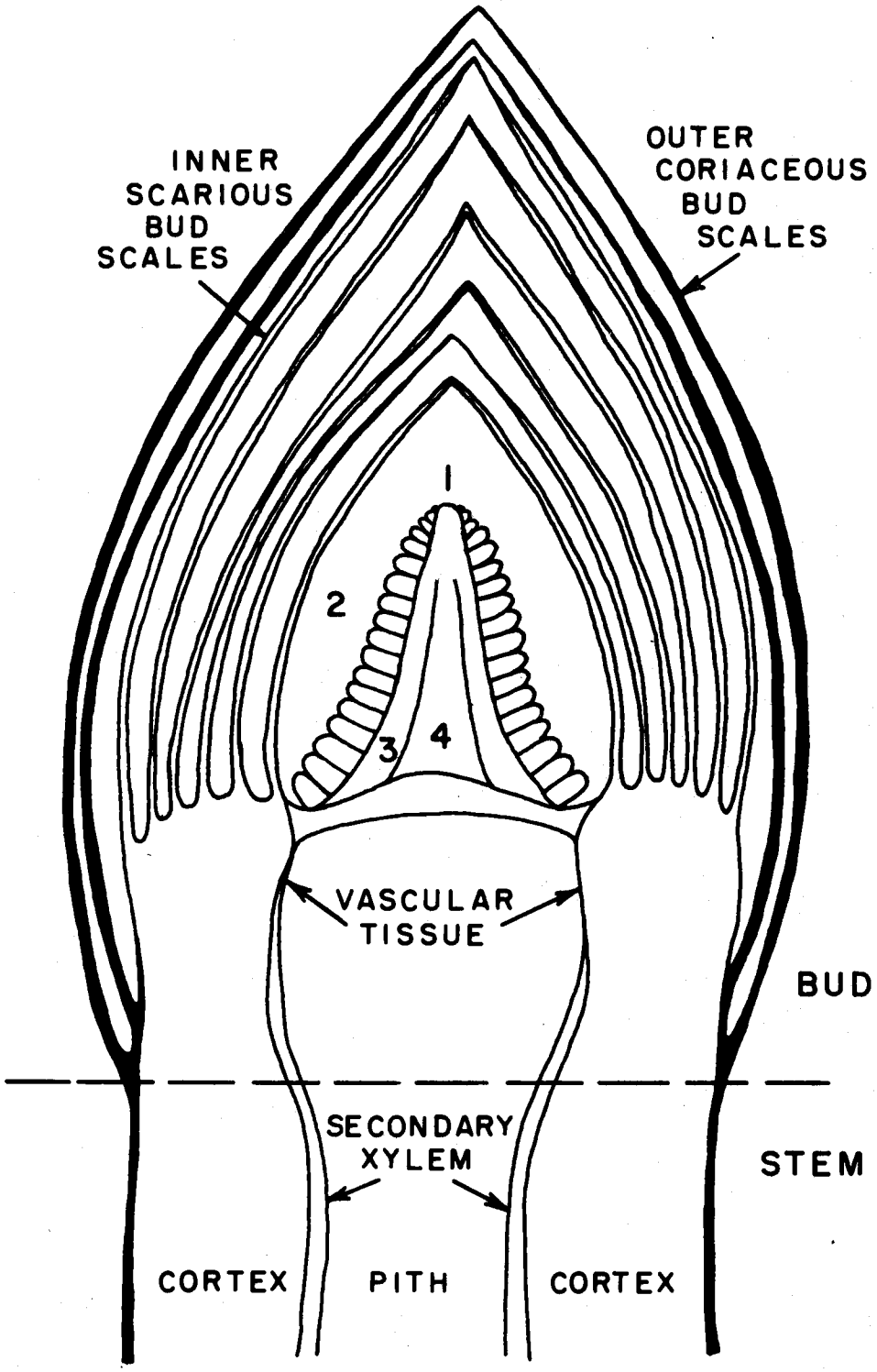

Figure 4. Schematic drawing of a median longitudinal section through a dormant bud and stem of Douglas-fir.

1. Meristematic tissues
2. Leaf primordia
3. Provascular tissue (provascular strands)
4. Rib region (future pith)
5. Crown

Table I. Percent of twigs uninjured, injured[1/] and killed[2/] by subfreezing temperatures ten days after treatment.

Date	Temperature	Potassium-deficient Trees				Potassium-nondeficient Trees			
		No. of Twigs	Uninjured	Injured	Killed	No. of Twigs	Uninjured	Injured	Killed
7-15	-7.5° C	24	8	25	67	24	21	4	75
7-18	-6.0° C	48	46	29	25	48	46	33	21
7-22	-6.5° C	48	2	31	67	48	6	75	19
8-22	-7.5° C	48	10	71	19	48	25	60	15

[1/] Needles did not show evidence of injury while stems were killed in July; both needles and stems were injured in August.

[2/] All tissues were killed.

not occur until mid-October (Figures 5 through 13) after minimum daily temperatures declined below 5° C. Mild frosts occurred on the 11th and 14th of October but ice probably did not form in tissues of the twig. These observations agreed with van den Driessche's (1969b) findings that temperatures as high as 7.5° C at night promoted hardiness of two-year-old Douglas-fir seedlings when day temperatures were 24° C but five days of mild frosts at -1° C for five hours duration had no additional effect. He found that photoperiods reduced from 16 hours to 12 hours also promoted hardiness.

Tissues of the stem and buds reached maximal resistance to freezing injury after subfreezing temperatures occurred each night from November 26 to December 6. Day and night temperatures during this period averaged 5° C and -3.5° C at Corvallis. Tissues of the needles did not reach maximal resistance until January, however (Table II). Rigidity of the foliage and absence of the characteristic first subcooling point of twigs frozen immediately after returning to the laboratory indicated that ice had formed in tissues outdoors for the first time. Except for seven days in early January when minimum daily temperatures averaged -3° C, frosts were mild and infrequent. Minimum daily temperatures seldom exceeded 7° C, however, until after the last frost on May 7.

Difference in susceptibility of various tissues to injury and temperatures that produced initial and complete injury was highest during the period of maximal resistance (Figures 5 through 13). Variation in injury between twigs from the same tree and between trees from the same population was least during this period.

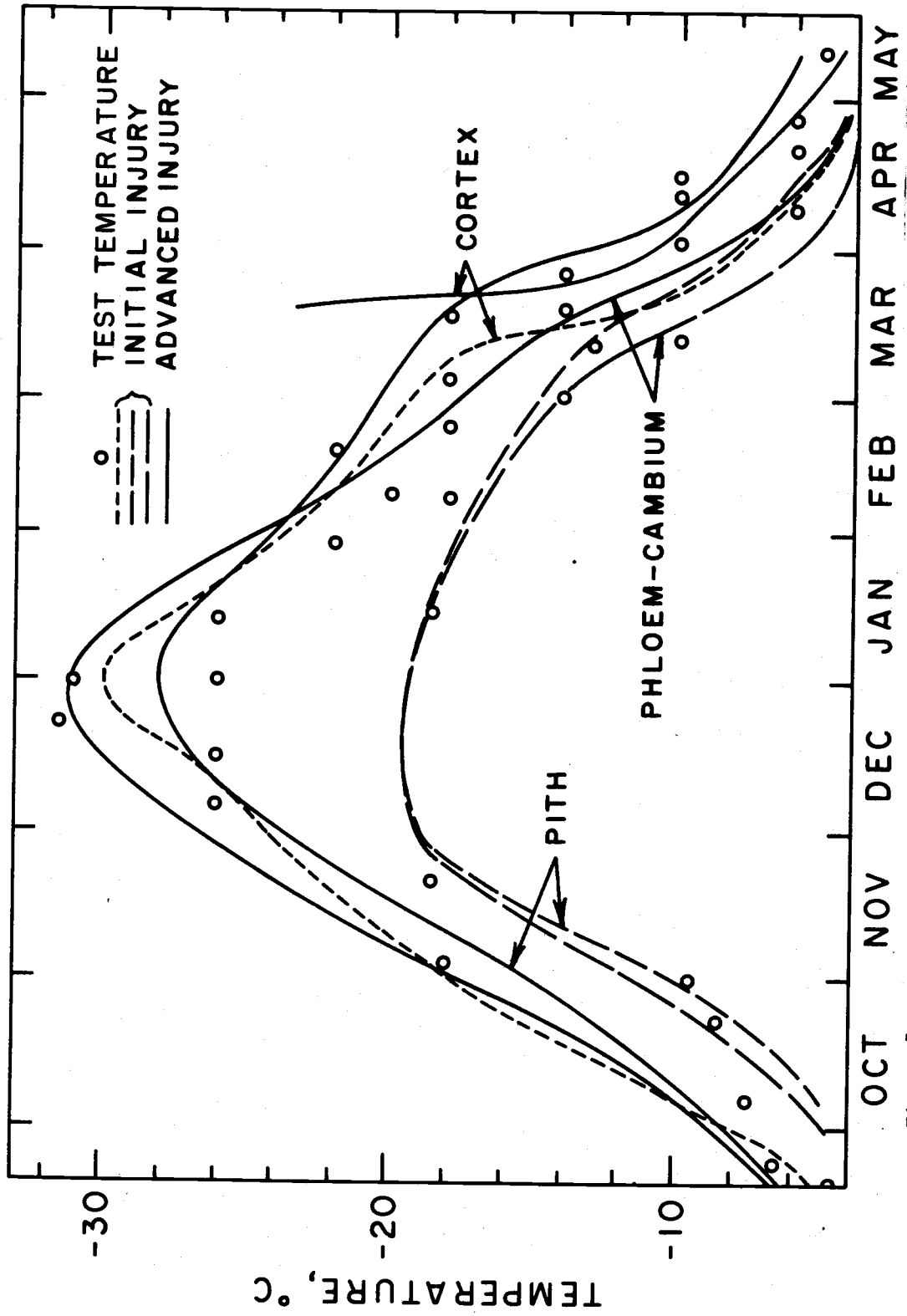

Figure 5. Seasonal variation in freezing resistance of tissues of the stem of potassium-nondeficient Douglas-fir.

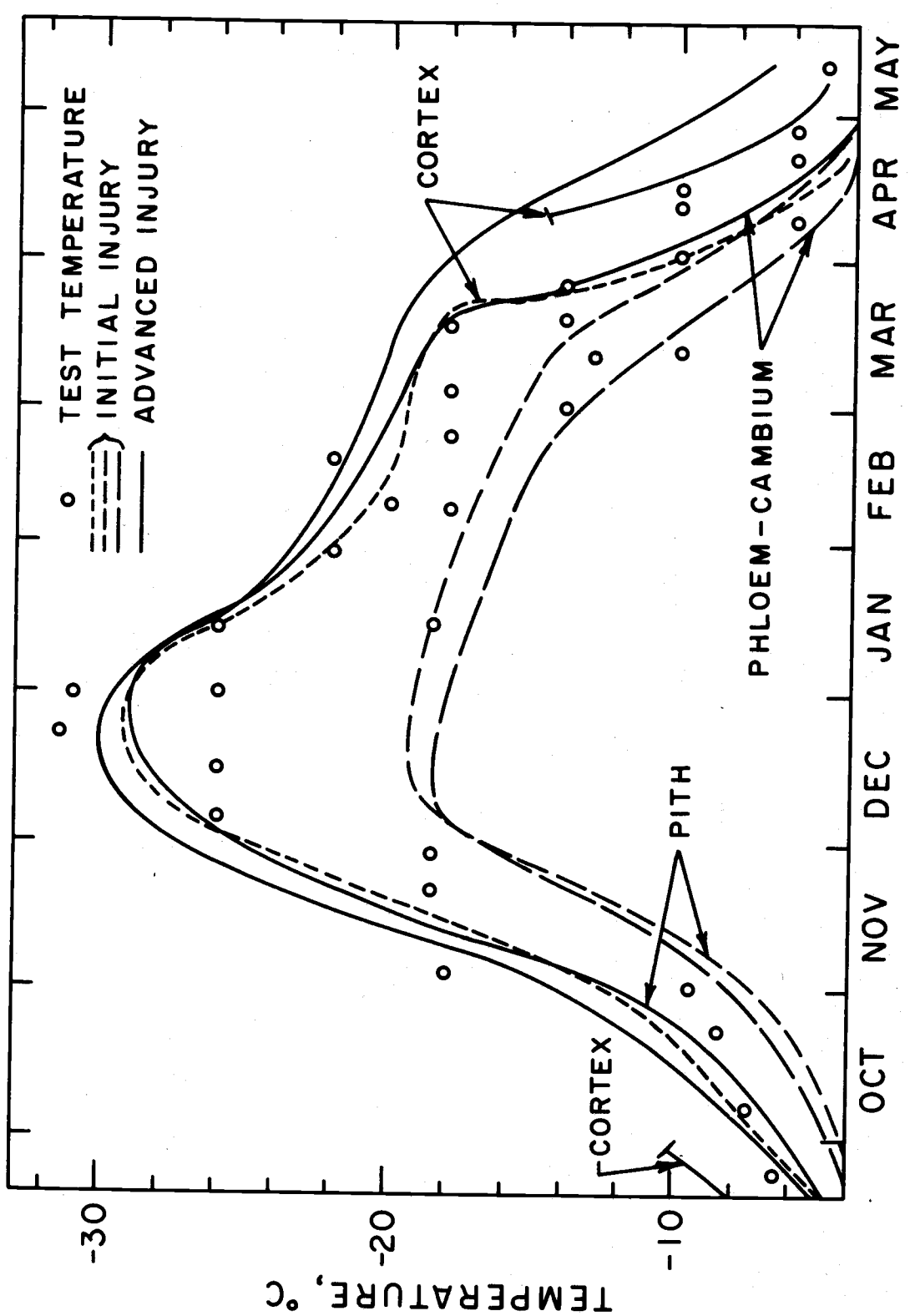

Figure 6. Seasonal variation in freezing resistance of tissues of the stem of potassium-deficient Douglas-fir.

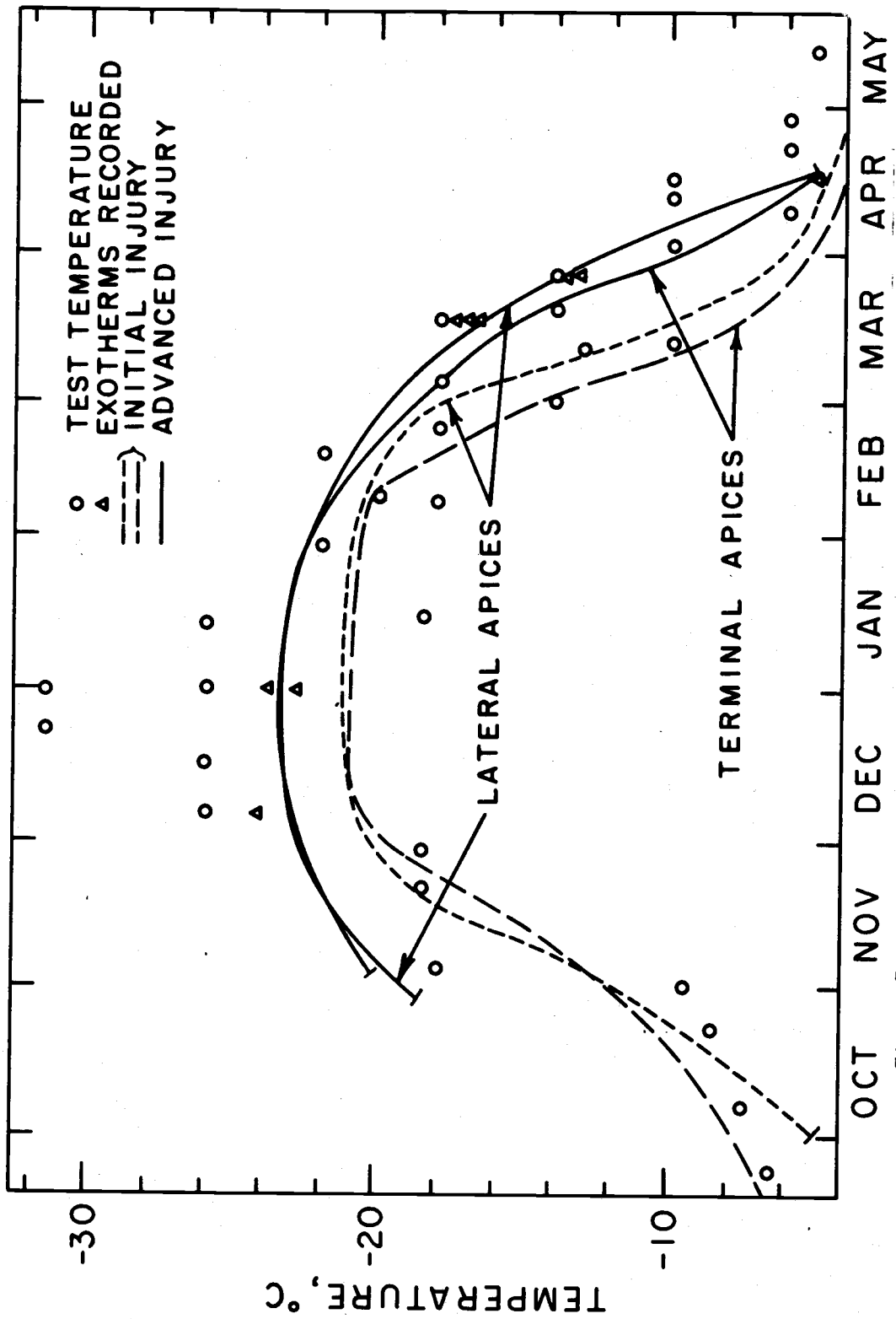

Figure 7. Seasonal variation in freezing resistance of vegetative apices of potassium-nondeficient Douglas-fir.

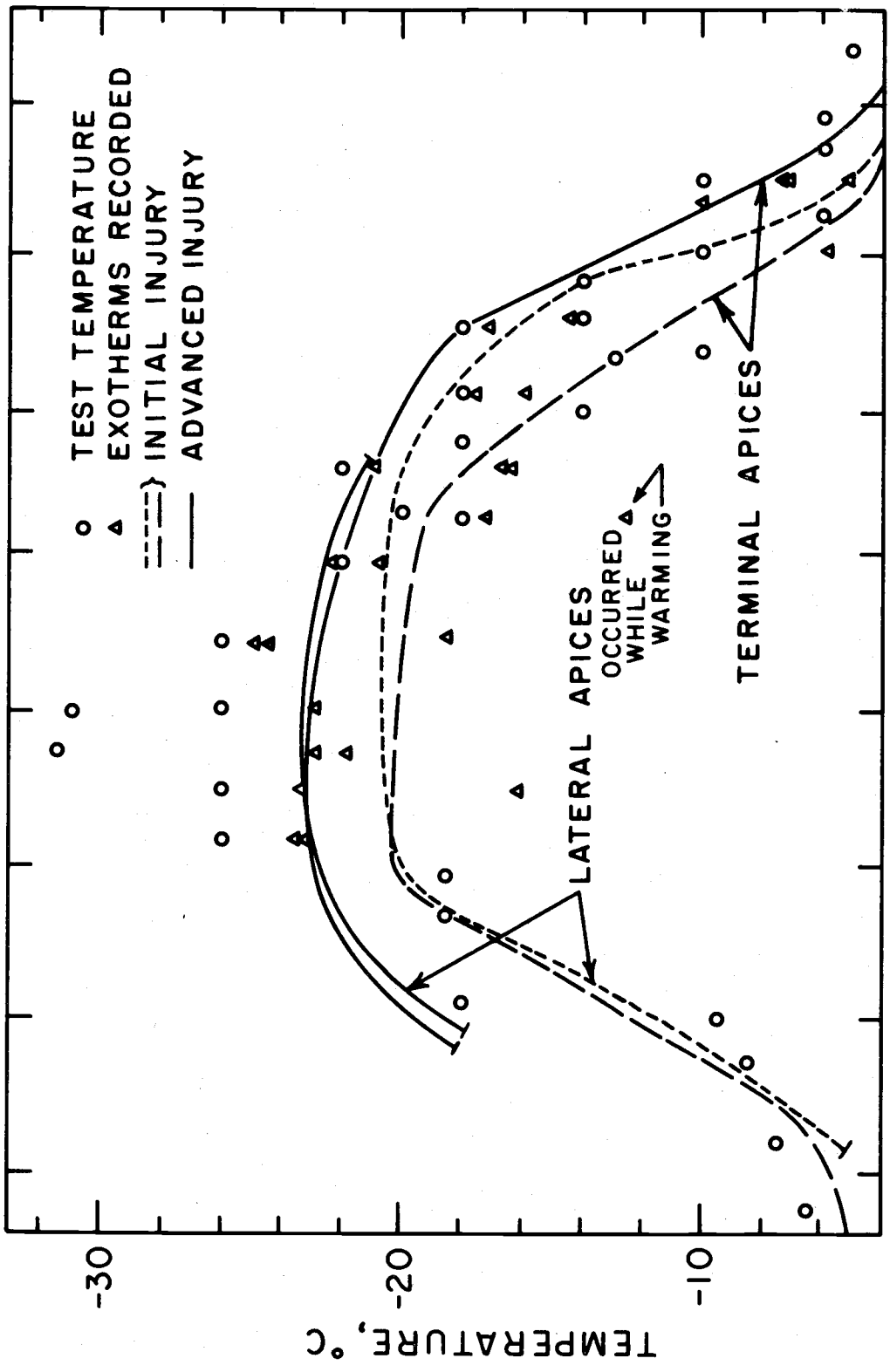

Figure 8. Seasonal variation in freezing resistance of vegetative apices of potassium-deficient Douglas-fir.

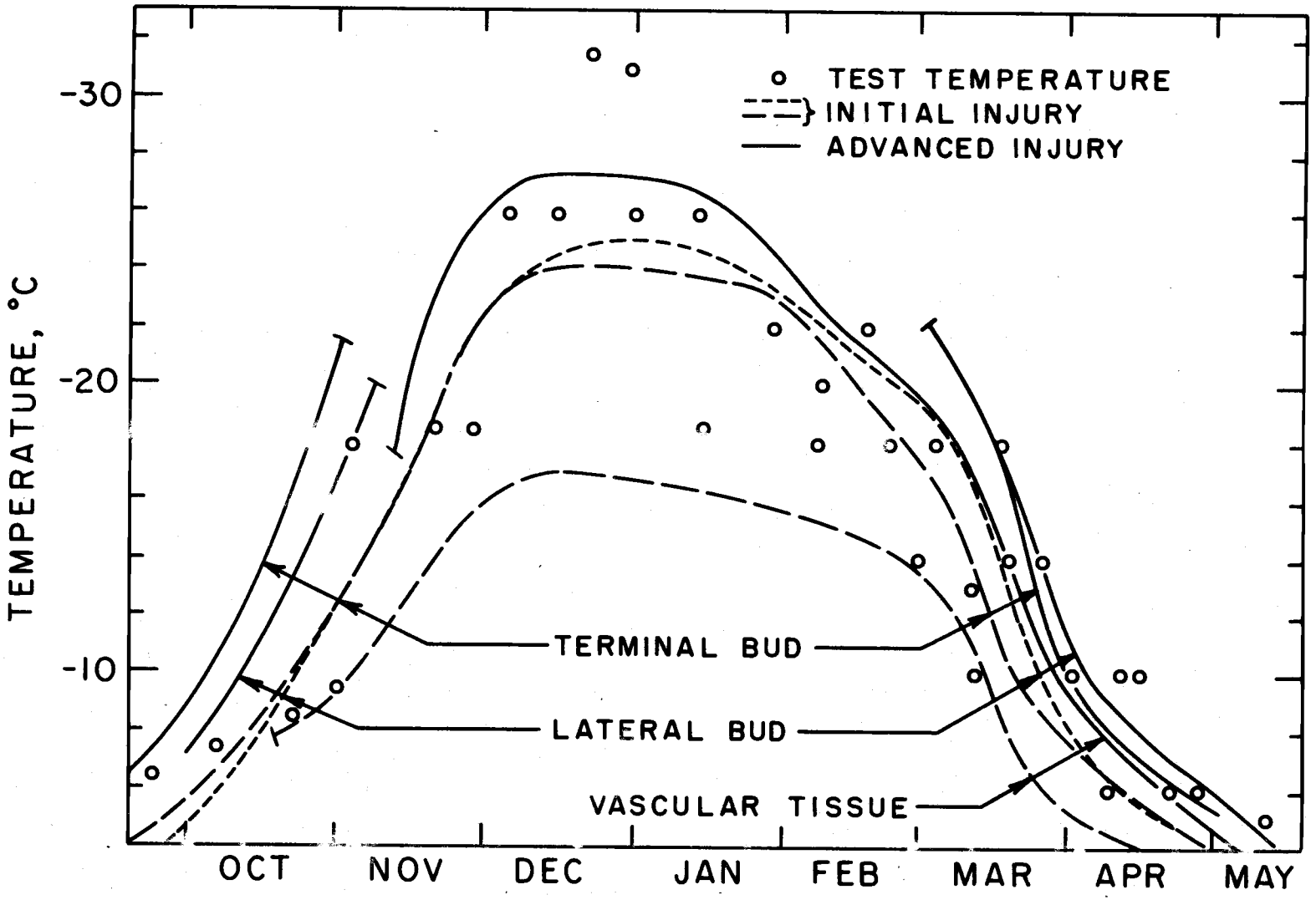

Figure 9. Seasonal variation in freezing resistance of tissues under the crown of vegetative apices of potassium-nondeficient Douglas-fir. Vascular tissue is the bud trace.

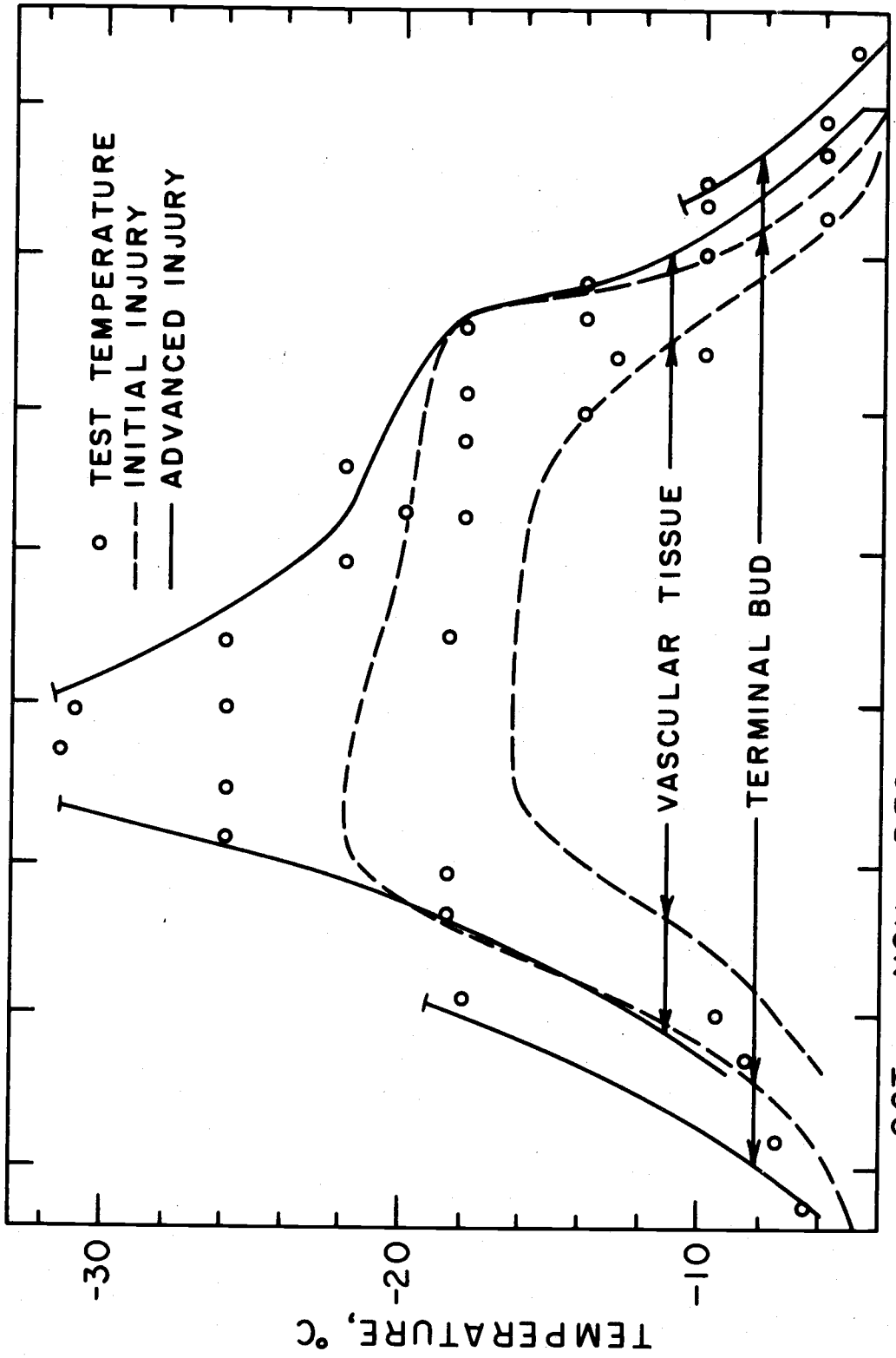

Figure 10. Seasonal variation in freezing resistance of tissues under the crown of vegetative apices of potassium-deficient Douglas-fir. Vascular tissue is the bud trace.

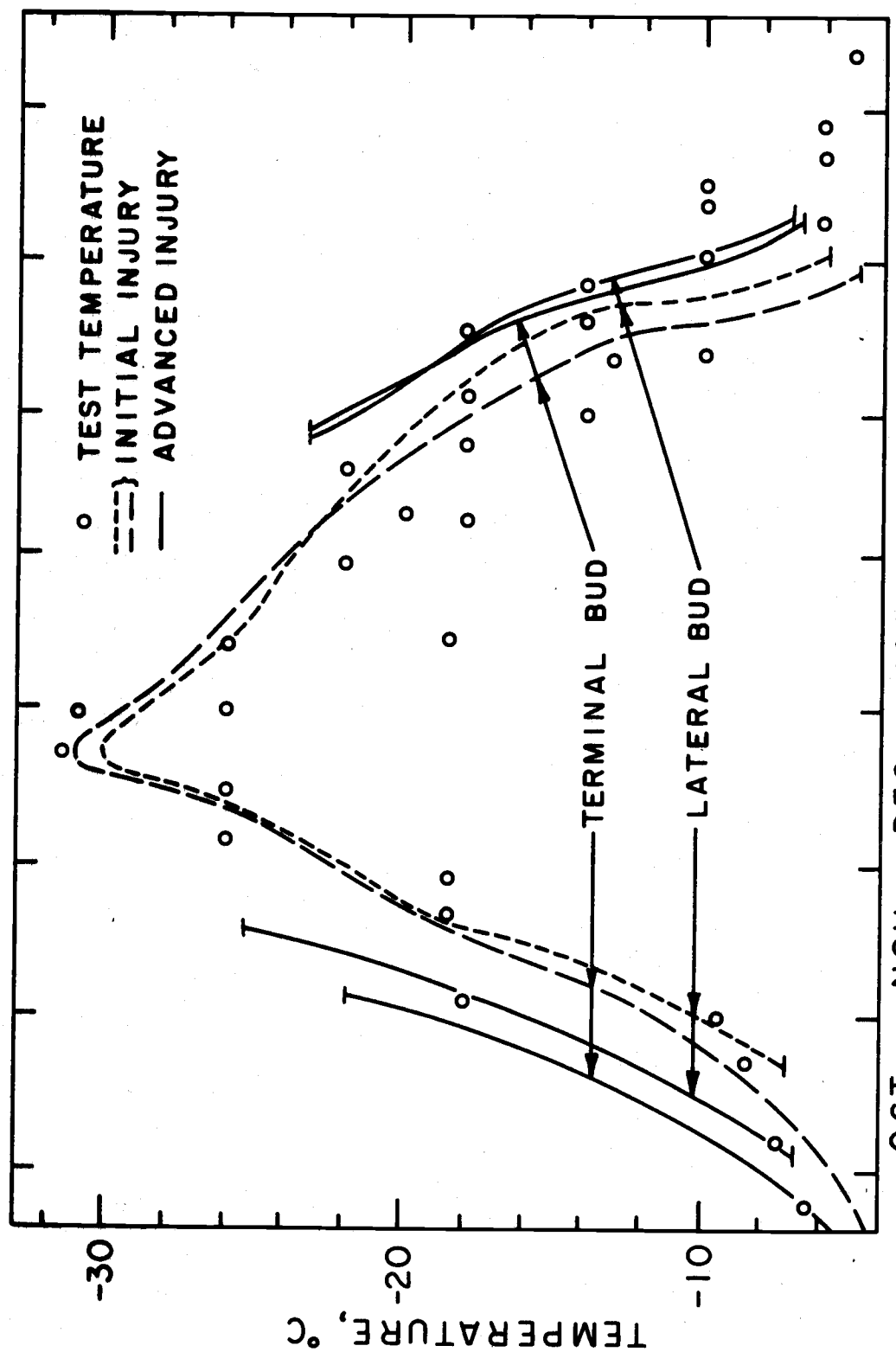

Figure 11. Seasonal variation in freezing resistance of the bud scales of potassium-nondeficient Douglas-fir.

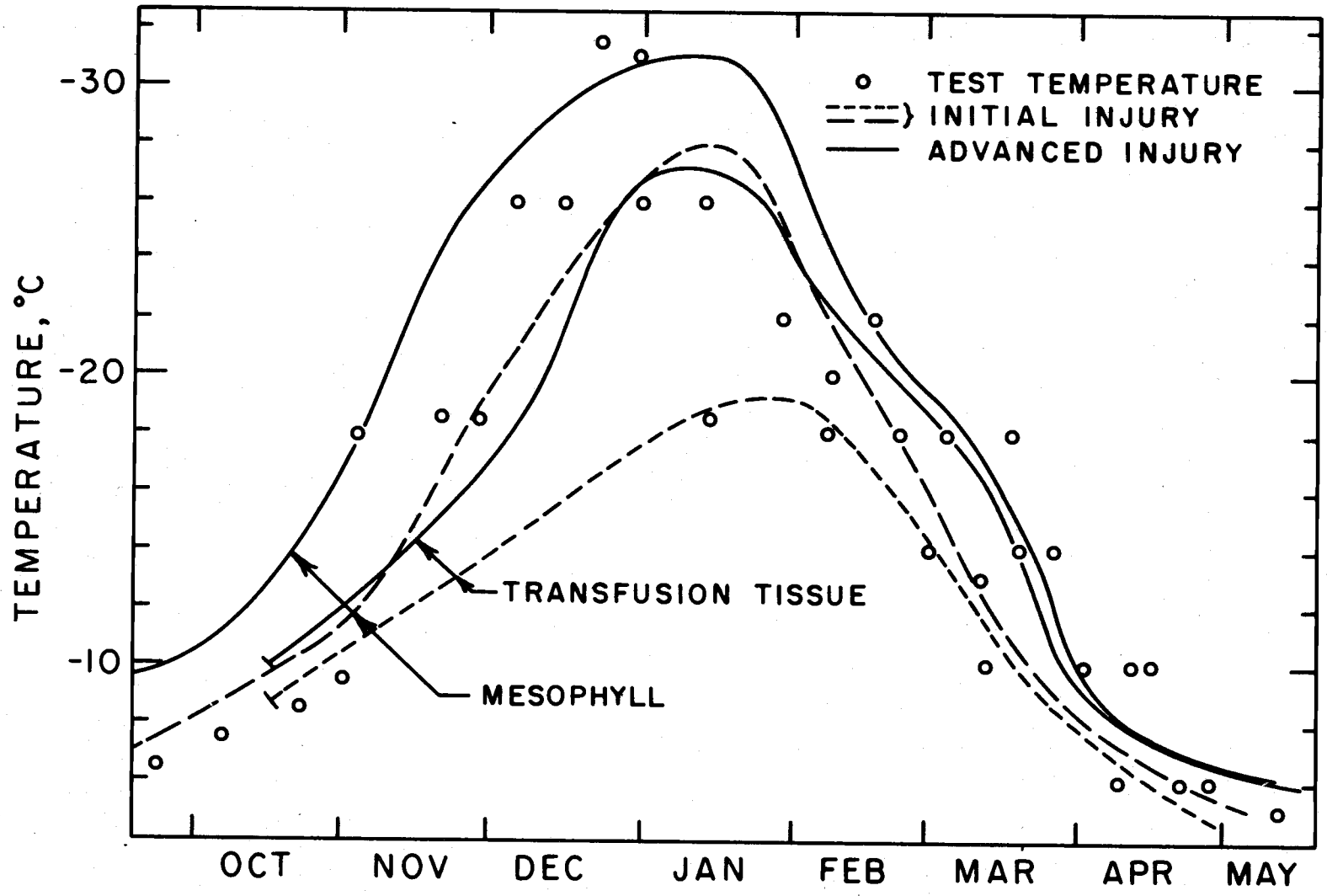

Figure 12. Seasonal variation in freezing resistance of tissues of the needle of potassium-nondeficient Douglas-fir.

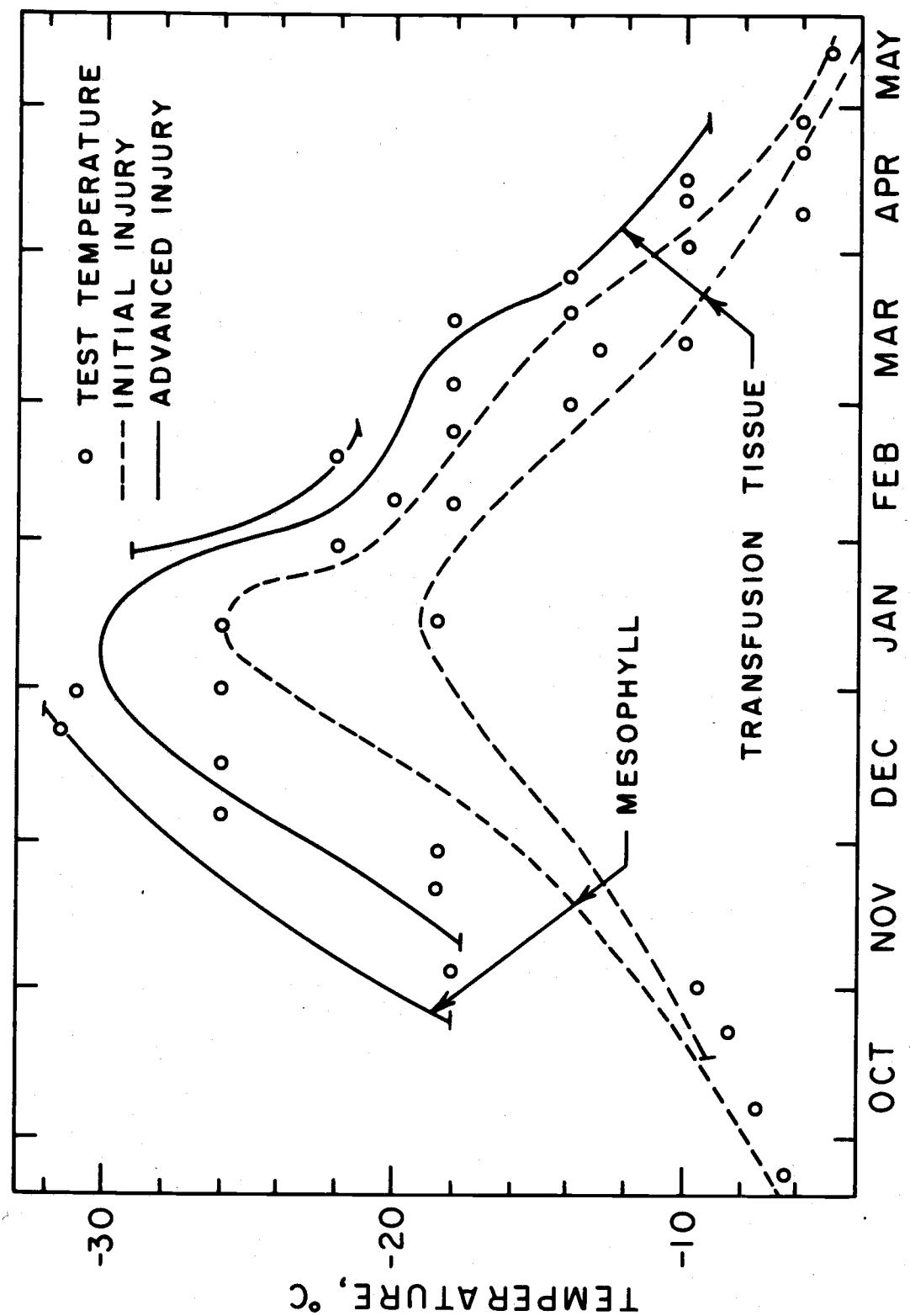

Figure 13. Seasonal variation in freezing resistance of tissues of the needle of potassium-deficient Douglas-fir.

Table II. Date of maximal resistance and lethal temperature (LT) that produced initial injury (damage index (Di) 1.25), partial injury (damage index (Di) 2.00) and complete death (damage index (Di) 3.00) of several tissues in the buds, stems and needles of Douglas-fir trees from potassium-deficient and nondeficient areas.

Tissue	Potassium-nondeficient Trees				Potassium-deficient Trees			
	Date	Di 1.25	Di 2.00	Di 3.00	Date	Di 1.25	Di 2.00	Di 3.00
Stem								
Cortex	12/31	-30° C	<-32° C	---	12/31	-29° C	<-32° C	---
Phloem-cambium	12/15	-19° C	-31° C	<-32° C	12/15	-18° C	-30° C	<-32° C
Pith	12/15	-19° C	-28° C	<-32° C	12/31	-19° C	-28° C	<-32° C
Bud								
Term. apices	12/1 to 2/1	-21° C	-23° C	-24° C	12/1 to 2/1	-20° C	-23° C	-25° C
Lat. apices	12/1 to 2/1	-21° C	-23° C	-24° C	12/1 to 2/15	-20° C	-23° C	-25° C
Vascular tissue of the trace	12/15	-17° C	-27° C	<-32° C	12/15	-16° C	-32° C	---
Term. bud scales	12/20	-31° C	-32° C	---	12/10 to 1/15	-27° C	-32° C	---
Lat. bud scales	12/20	-30° C	-32° C	---	12/10 to 1/15	-26° C	-32° C	---
Tissues of the stem enclosed by coriaceous bud scales								
Terminal	12/10 to 2/1	-24° C	<-32° C	---	12/10 to 2/1	-22° C	<-32° C	---
Lateral	12/10 to 2/1	-25° C	<-32° C	---	12/10 to 2/1	-22° C	<-32° C	---
Needle								
Transfusion tissue	1/15	-19° C	-27° C	-32° C	1/15	-19° C	-30° C	<-32° C
Mesophyll	1/15	-28° C	-31° C	<-32° C	1/15	-26° C	<-32° C	---

Stem

Cortical tissue was more resistant than other tissues of the stem until after growth processes resumed in early spring, and living cells of the pith were most resistant (Figures 5 and 6). The pith developed greater discoloration in the autumn (Figures 14, 15 and 16), however, and appeared more susceptible to injury than tissues of the phloem-cambium region. The pith was often most severely injured opposite lateral buds (Figure 17) during development of freezing resistance.

Browning in the secondary xylem of the stem reflected injury in adjacent tissues. Injury to the secondary xylem was not reported although parenchyma cells of rays were susceptible to injury (Figure 18). Apparently products of oxidation that caused browning after injury to cells of the pith and phloem-cambium tissues were absorbed by cell walls of the tracheids (Figure 19). However, the tracheids in transverse section of injured stems changed abruptly from brown to white while drying under the microscope because cell walls appeared to suddenly contract and split longitudinally to expose a white crystalline internal structure of the secondary wall (Figures 3 and 20).

The first noticeable change in relative resistance of tissue in the stem of potassium-nondeficient Douglas-fir appeared in January when the phloem-cambium region lost resistance more rapidly than the pith and cortex. Phloem-cambium tissues of potassium-nondeficient Douglas-fir were most susceptible to injury after this time (Figures 2, 5 and 21). The same change in resistance occurred in early

Figure 14. Median longitudinal and oblique transverse sections of buds frozen to -6.5° C on September 23. Damage index: Left: terminal bud that was not frozen. Center: terminal bud, scales, 2.00 (base of inner bud scales); apex, 1.00; pith, 2.00 (water-soaked); vascular tissue, 2.00; cortex, 1.50; pith of stem, 3.00; xylem of stem, 2.00; phloem-cambium of stem, 3.00; cortex of stem, 1.50. Right: lateral bud, pith, 3.00; vascular tissue, 3.00; cortex, 2.00. Note complete injury in the pith of the stem below the bud. Inner bud scales and pith at the base of the bud were among the last tissues to harden in the autumn. 3x.

Figure 15. Transverse and longitudinal sections of stems frozen to -18° C on November 3 (right) and not frozen (left). The stems were from the same tree about 2 cm from terminal buds. Damage index: pith, 3.00; xylem, 3.00; phloem-cambium, 2.50; cortex, 1.50. Note complete injury in the pith of the stem. 6x.

Figure 16. Transverse and longitudinal sections of stems frozen to -18° C on November 3 (left) and not frozen (right). The stems were from the same tree about 2 cm from terminal buds. Damage index: pith, 3.00; xylem, 3.00; phloem-cambium, 3.00; cortex, 2.00. 6x.

Figure 17. Median longitudinal sections of lateral buds and stems frozen to -18° C on November 3 (left and center) and not frozen (right). The stems were from the same tree. Damage index: buds, scales, 1.50; apices, 1.00; pith, 3.00; vascular tissue, 3.00; cortex, 2.00 (right), 1.00 (left); stem, pith, 2.50; xylem, 1.50; phloem-cambium, 2.00; cortex, 1.00. Note extensive injury in the pith of stems opposite the lateral buds. 6x.

Figure 18. Tangential section through the xylem of a bud trace frozen to -18° C on February 7. Injury is evident in at least two uniserate rays. Damage index: 2.00. 240x.

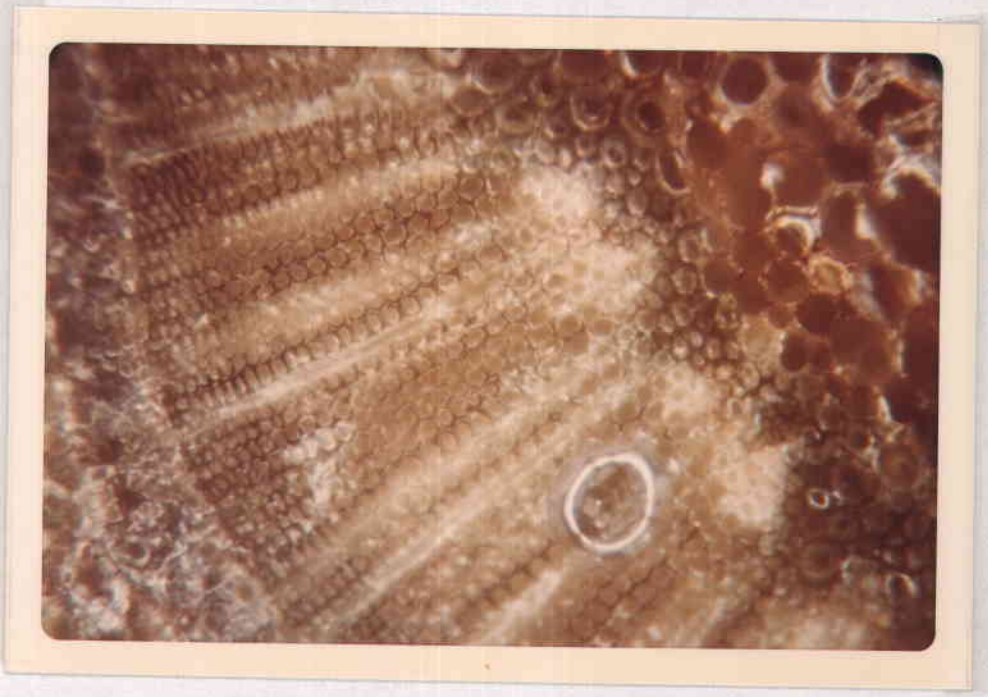

Figure 19. Transverse section of a stem frozen to -22° C on January 29. Damage index: pith, 2.00; xylem, 2.50; phloem-cambium, 2.00. Dark appearance of the cell walls of tracheids was characteristic of injured twigs before the xylem dried. 90x.

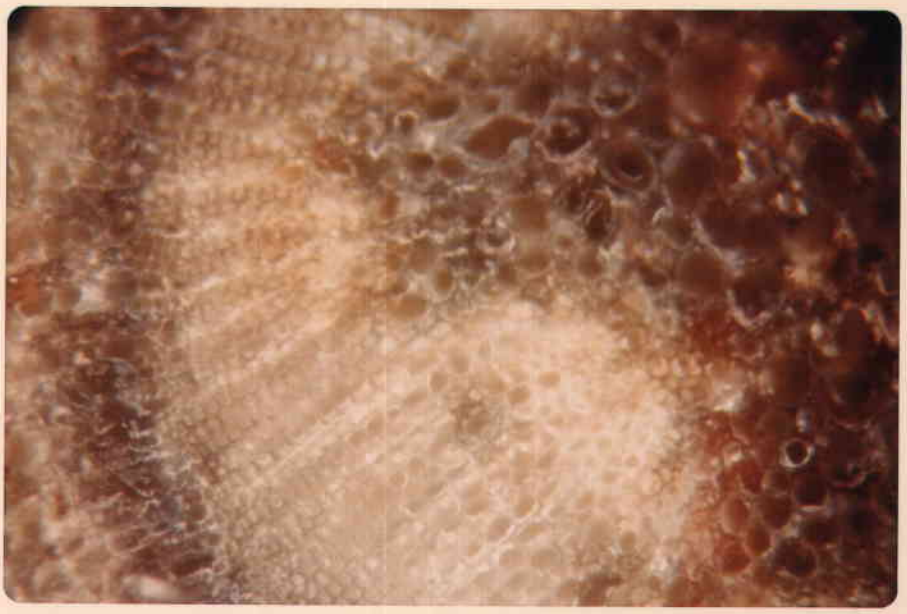

Figure 20. Transverse section of a stem frozen to -22° C on January 29. Damage index: pith, 2.50; xylem, 2.50; phloem-cambium, 2.00; cortex, 1.00. Note discoloration of the primary xylem from injured pith parenchyma and white crystalline structure of secondary walls of tracheids that dried. 90x.

Figure 21. Transverse and longitudinal sections of stems frozen to -26° C (right) and -18° C (center) on January 13 and 14, and not frozen (left). The stems were from the same tree. Damage index: Right: -26° C, pith, 2.25; xylem, 2.00; phloem-cambium, 2.50; cortex, 1.00. Center: -18° C, pith, 1.50; xylem, 1.50; phloem-cambium, 2.00; cortex, 1.00. Note extensive injury in the phloem-cambium region. 6x.

December for potassium-deficient trees when cells of the phloem-cambium region failed to develop the same resistance to initial injury as parenchyma of the pith (Figure 6).

The wide dark band in the phloem of Figure 2 was characteristic of partial injury in this region before loss of resistance was substantial. Evaluation of initial injury to the phloem-cambium region was more difficult in late winter because uninjured phloem was dark from increased metabolic activity and translocation. Injury to the phloem-cambium in relation to other tissues of the stem became more severe and evident as spring approached, however (Figures 22 and 23). Variation in injury between twigs from different trees also increased and twigs with the greatest enlargement of the vegetative apices at room temperature were least resistant.

Bud

Tissues of the stem within the outer coriaceous bud scales were most severely injured under the crown tissue of terminal and lateral apices before freezing resistance increased in October. Frequent collapse of all cells of the pith under the crown tissue indicated that formation of ice was massive in this region (Figures 24 and 25). Massive formation of ice may have caused severe dehydration and partial injury of vegetative apices leading to a bleached appearance in tissues external to the procambium on the periphery of the apex (Figure 26). Partial injury was also caused by leaching of toxic substances from dead bud scales into the periphery of the vegetative

Figure 22. Transverse section of stems frozen to -4.5° C on May 5 (right) and not frozen (left). The stems were from the same tree. Damage index: pith, 1.00; xylem, 2.00; phloem-cambium, 2.50; cortex, 1.75. Note high susceptibility of the phloem-cambium region and injury in the cortex. 6x.

Figure 23. Transverse section of stems frozen to -10° C on April 11 (right) and not frozen (left). The stems were from the same tree. Damage index: pith, 1.00; xylem, 2.25; phloem-cambium, 3.00; cortex, 2.50. Note the dead phloem-cambium region and excessive injury in the cortex. 6x.

Figure 24. Longitudinal sections of terminal buds frozen to -6.5° C on September 23 (center and right) and not frozen (left). Damage index: scales, 2.00; apex, 1.50; pith, 3.00; vascular tissue, 3.00; cortex, 2.00. Note complete destruction of cells in the pith under the crown. 3x.

Figure 25. Median longitudinal sections of terminal buds frozen to -18° C on November 3 (center and right) and not frozen (left). The buds were from the same tree. Damage index: Center: scales, 1.50 (outer coriaceous scales are dead); apex, 1.00; pith, 3.00; phloem-cambium, 3.00; cortex, 1.50; Right: scales, 1.50; apex, 1.00; pith, 2.25; phloem-cambium, 2.00; cortex, 1.50. Complete destruction of cells in the pith under the crown was frequent in unhardened twigs. 6x.

Figure 26. Median longitudinal section of terminal buds frozen to -9.5° C on October 31 (left) and not frozen (right). The buds were from the same tree. Damage index: scales, 2.00; apex, 2.00; pith, 2.00; vascular tissue, 2.50; cortex, 2.00. Note injury in the periphery of the apex. 3x.

apex and not from formation of ice before freezing resistance developed in immature buds.

Severe browning at the point of initiation of inner scarious bud scales and in the pith about 3 mm below the crown indicated that these tissues were among the most susceptible to injury in autumn and last to mature (Figures 14, 26 and 27). Formation of ice in the pith enclosed by coriaceous bud scales may dehydrate underlying pith tissue and delay injury during mild frosts (Figure 14).

Tissues of lateral buds were injured more severely than tissues of terminal buds until early November for potassium-nondeficient trees, and until early December for potassium-deficient trees (Figures 7 through 9). Lateral buds of Douglas-fir are initiated at the onset of shoot growth in the spring and their development is two or three weeks behind development of the terminal bud (Owens, 1968). The difference in development and freezing resistance was not apparent in late fall after most leaves were initiated in the vegetative apices but lateral buds were smaller and had fewer leaf primordia than terminal buds. Tissues of lateral buds were more resistant than tissues of terminal buds from late autumn until loss of hardiness was complete in the spring. The small size of lateral apices may have enhanced movement of water to underlying tissues and may have reduced the subcooling temperature.

Bud scales and tissues under the crown enclosed by coriaceous bud scales were more resistant than the vegetative apices and tissues of the stem after twigs reached maximal resistance to freezing in

Figure 27. Median longitudinal section of terminal buds frozen to -9.5° C on October 31 (left) and not frozen (right). The buds were from the same tree. Damage index: scales, 1.50; apex, 1.00; pith, 2.00; vascular tissue, 1.00; cortex, 1.00. Pith of the stem under the bud and inner scales were particularly susceptible to injury in the autumn. 6x.

December (Figures 28 and 29). These tissues enclosing the vegetative apex and crown remained more hardy until resistance was lost in early spring. A gradual shift from green to white in uninjured cells of the pith and cortex indicated that chlorophyll was lost as these tissues matured and hardened. Tissues at the base of the inner bud scales, pith and vascular traces under the crown, were often water-soaked in appearance after freezing. The water-soaked condition may have represented an accumulation of free water in extracellular spaces from formation of ice without injury because moisture stress was absent while twigs were in water during recovery.

The vegetative apices maintained a uniform level of freezing resistance in December and January, but lost resistance slowly in February (Figures 7 and 8). Loss of resistance became rapid in March as growth processes accelerated. The vegetative apex was always dead after ice formation during the period of greatest hardiness although a few cells of the future pith under the rib meristem remained green and may not have been disrupted by ice (Figure 29). Death could be recorded by measuring the latent heat of fusion of water in the vegetative apex. The number of exotherms per twig recorded after ice formed in the stem agreed precisely with the number of dead vegetative apices within 5 mm of a thermocouple inserted into the pith of a stem under the terminal bud (Table III).

Injury to cells of the crown between the vegetative apex and pith may have been prevented by dehydration as ice formed in the pith. Cells of this tissue were dense, had thick walls and often

Figure 28. Longitudinal section of lateral buds frozen to -26° C (left) and -18° C (center) on January 13 and 14 and not frozen (right). The buds were from the same tree. Damage index: Left: -26° C, scales, 1.00; apex, 3.00; pith, 2.00; vascular tissue, 2.00; cortex, 1.50; trace, 2.00. Center: -18° C, scales, 1.00; apex, 1.00; pith, 1.50; vascular tissue, 1.50; cortex, 1.00; trace, 1.50. Note that the scales and tissues directly under the crown were highly resistant to freezing injury in early winter. 6x.

Figure 29. Median longitudinal sections of terminal buds frozen to -26° C on January 13 (left) and not frozen (right). The buds were from the same tree. Damage index: scales, 2.00; apex, 3.00; pith, 2.00; vascular tissue, 2.25; cortex, 2.00. Stem: pith, 2.50; xylem, 2.50; phloem-cambium, 3.00; cortex, 2.25. Note that the scales and tissues directly under the crown are highly resistant to freezing injury in early winter. A few cells in the vegetative apex appeared uninjured. 6x.

darkened after the vegetative apex was killed but appeared uninjured otherwise.

Table III. Number of uninjured and dead vegetative apices 5 mm from a thermocouple and exotherms registered per twig frozen at about 1° C per minute in November.

No. of Twigs	No. of Exotherms	No. of Veg. Apices	No. of Veg. Apices		Average Test Temp.
			Uninjured	Dead	
8	3	3	0	3	-16° C
3	2	2	0	2	-15° C
3	2	3	1	2	-16° C
5	1	3	2	1	-16° C
4	1	2	1	1	-16° C
3	0	2	2	0	-13° C
3	0	3	3	0	-13° C

Vegetative apices were partially injured after growth processes resumed in late February and early March. Death of cells in the rib region above the crown and between the provascular bundles left a black region that enlarged into a cavity in the future pith as surrounding tissues grew. Intercellular ice did not cause partial injury of this type. Previous trials had shown that formation of ice was complete in all tissues of the vegetative apex in one minute or less after nucleation. Nucleation must have taken place in the rib region if formation of ice was the direct cause of death because surrounding cells were not discolored and were capable of growth after the freezing test. Movement of ice from underlying tissues into the region

of injury through undeveloped cell walls of the same type of cell without causing formation of intracellular or intercellular ice seemed unlikely.

Isolated injury to the rib region of vegetative apices was not observed after buds began to swell in early spring but injury was apparent in the meristematic region and adjacent leaf primordia (Figures 30 and 31). This injury was not the direct result of ice formation because it was observed occasionally in unfrozen controls. Subfreezing temperatures, or destruction of vascular tissue from ice formation in the stem enclosed by coriaceous bud scales, or both, may have accelerated breakdown processes in growing apices that were injured. In addition, injury may have occurred in the field before the twigs were collected. Subfreezing temperatures or injury to tissues of the stem also inhibited growth of those apices that showed no evidence of injury because buds of frozen twigs did not enlarge while new growth emerged from buds of unfrozen twigs at room temperature.

Tissues of the vascular region of the bud trace were among the first to show initial injury during the period of greatest hardiness (Figures 9 and 10, Table II). Injury was evaluated for xylem as well as phloem tissue, and included a striking discoloration of the primary tracheids that absorbed products of browning from adjoining pith parenchyma and pith rays of the interfascicular region (Figures 32, 33, and 34). The phloem-cambium region of the bud trace, as that of the stem, dehardened rapidly in late winter and became more susceptible to freezing injury than the pith parenchyma.

Figure 30. Longitudinal sections of terminal buds frozen to -10° C on April 11 (right) and not frozen (left). The buds were from the same tree. Damage index: scales, 1.50; apex, 2.00; pith, 2.00; vascular tissue, 2.00; cortex, 1.25. Injury was occasionally evident in the unfrozen vegetative apex. 6x.

Figure 31. Longitudinal sections of terminal buds frozen to -10° C on April 11 (right) and not frozen (left). The buds were from the same tree. Damage index: scales, 1.00; apex, 2.00; pith, 1.25; vascular tissue, 1.00; cortex, 1.00. Stem: pith, 1.00; vascular tissue, 2.00; cortex, 1.00. Injury was occasionally evident in the unfrozen vegetative apex. 6x.

Needle

Injury to the mesophyll and tissues within the endodermis was evaluated during the course of study after the needles developed partial freezing resistance in the autumn. Severe injury was most conspicuous at the tips of both hardened and unhardened needles (Figure 35). Probably, ice nucleation occurred in the xylem that terminates as a few cells about 200 μ from the needle tip. Although the endodermis extends to the last xylem and phloem elements, it is barely distinguishable from the surrounding mesophyll in this region (Owens, 1968) and probably offered no resistance to growth of ice.

Tissues of the needle were injured or killed without external evidence of injury after they developed freezing resistance, as were tissues of the stem and bud, with exception of the vegetative apex. Transfusion tissue that contained transfusion parenchyma, transfusion tracheids and albuminous cells and enclosed the vascular bundle of the leaf (Figure 36) was the first tissue in the needle to show evidence of injury (Figure 37). Transfusion tracheids have empty lumina (Owens, 1968) and the deep discoloration on each side of the vascular bundle between the xylem and phloem probably resulted from injury to transfusion parenchyma and albuminous cells. Another reason why deep discoloration occurred in the transfusion tissue was that transfusion parenchyma contained more tannins and resins than other cells of the needle. Injury to parenchyma cells that radiate through the xylem may have stained the cell walls of tracheids that turned dark (Figure 37).

Figure 32. Longitudinal radial section of a bud trace frozen to -22° C on January 29. Damage index: pith, 1.75; vascular tissue (trace), 2.00. Brown discoloration in the primary xylem resulted from injury to pith ray parenchyma. 90x.

Figure 33. Transverse section of bud trace frozen to -22° C on January 29. Damage index: pith, 1.00; vascular tissue (trace), 2.00; cortex, 1.00. Brown discoloration in the xylem resulted from injury to pith ray parenchyma. 40x.

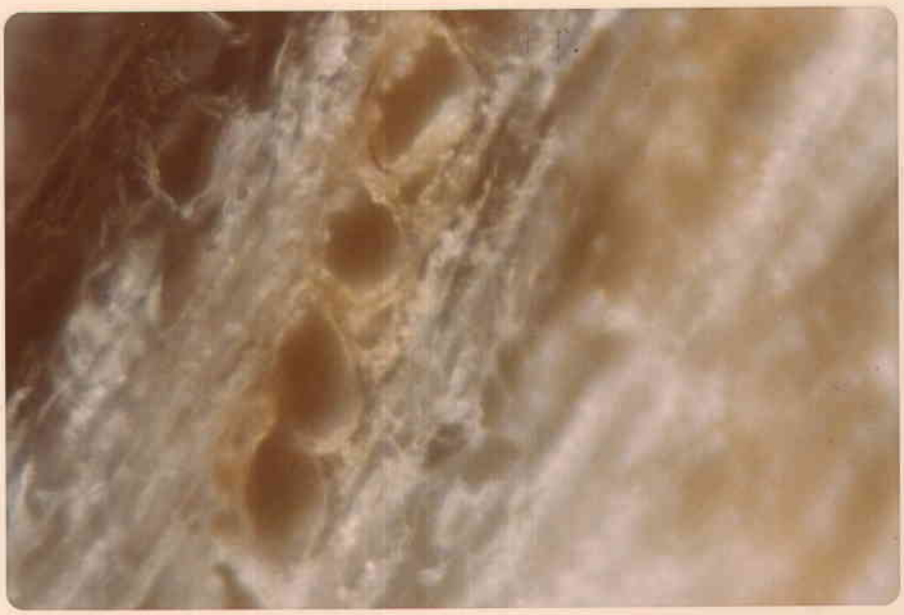

Figure 34. Tangential-radial section of a bud trace frozen to -18° C on February 7. Cell walls of pith ray parenchyma are discolored. Damage index: 2.00. 240x.

Figure 35. Injury to the tips of needles frozen to -6.5° C on September 23. 3x.

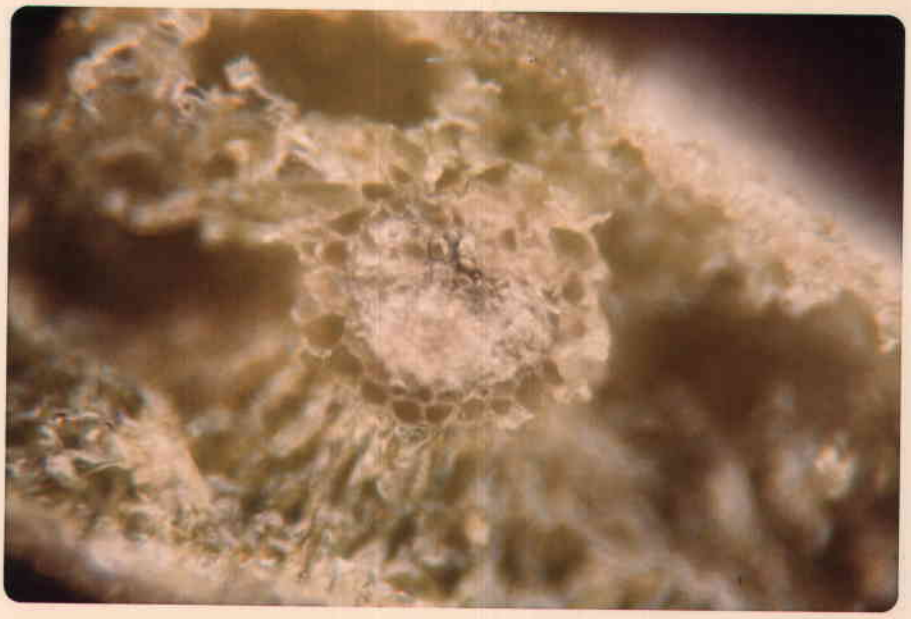

Figure 36. Transverse section of an unfrozen needle collected on January 29 and photographed on February 11. 80x.

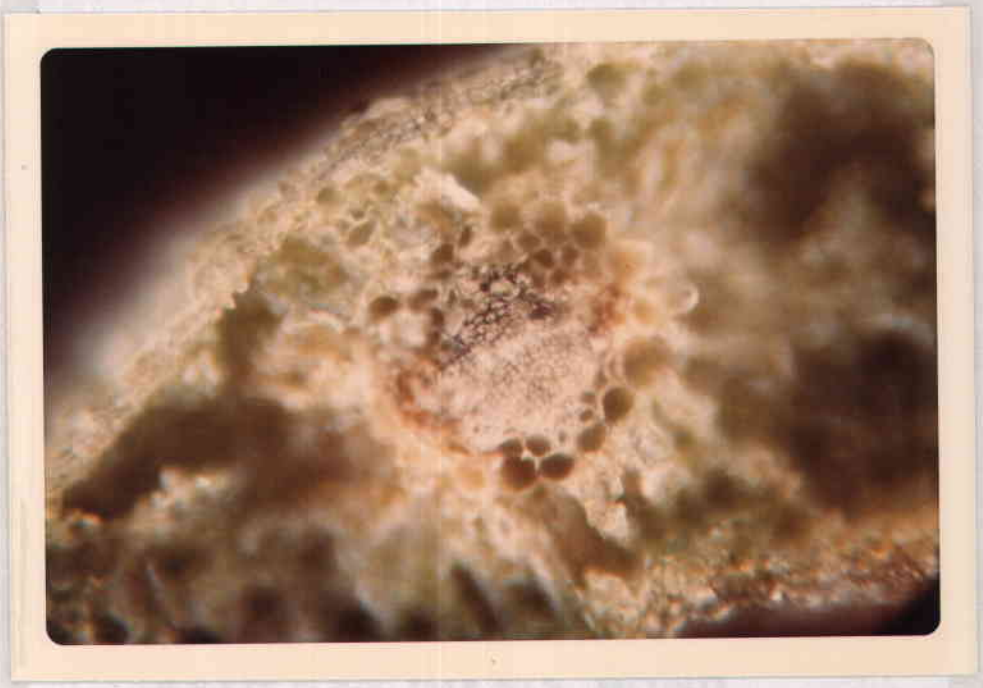

Figure 37. Transverse section of a needle frozen to -22° C on January 29. Damage index: transfusion tissue, 2.50; mesophyll, 1.00. Transfusion tissue on each side of the phloem was severely injured. 80x.

Discoloration of the mesophyll began with the cells adjoined to the endodermis and radiated outward along connected cells (Figure 38). The compact mesophyll that extends from the endodermis to the lower hypodermis, and the adjacent spongy mesophyll were less resistant to freezing injury than the palisade mesophyll on the adaxial side of the needle (Figure 39). Injury to the abaxial mesophyll appeared to be intensified by potassium deficiency. Injury to the transfusion tissue and abaxial mesophyll was frequently visible as a dark line along the midrib of the lower surface of the needle (Figure 40).

The endodermis apparently protects mesophyll tissues from ice formation. Salt and Kaku (1967) and Kaku and Salt (1968) observed that growth of ice from the xylem into the mesophyll was inhibited by the endodermis if the needles were dehydrated. Oxidized products from injured transfusion parenchyma may have diffused across the endodermis during the recovery period when moisture was ample.

Freezing trials in April, after the twigs had lost resistance, often caused needles to abscise from the stem without injury to the mesophyll. Loss of the needles appeared to be associated with injury to the cortex of the stem and needle trace.

Figure 38. Longitudinal section near the endodermis of a needle frozen to -22° C on January 29. Damage index: mesophyll, 1.50; transfusion tissue, 2.50. Mesophyll surrounding the endodermis was injured. 80x.

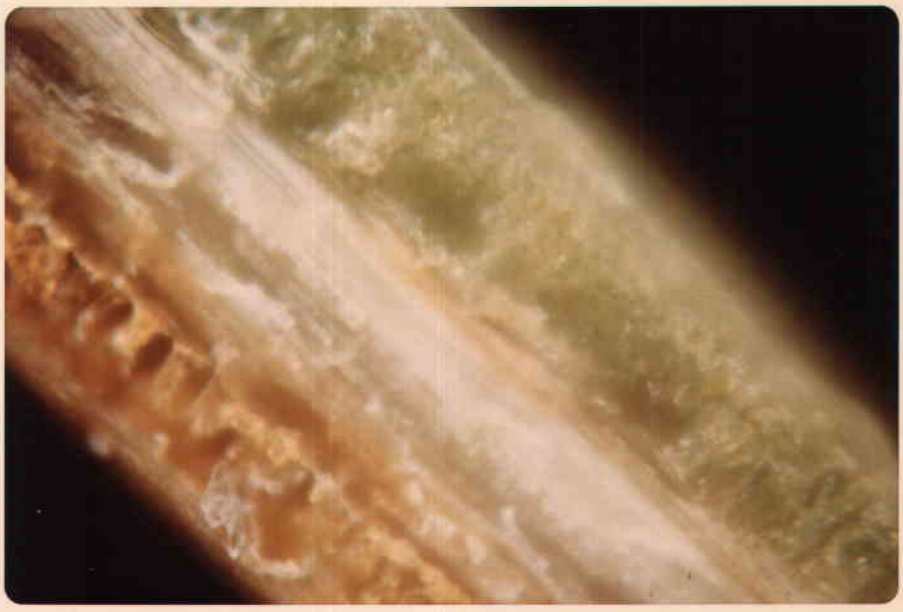

Figure 39. Longitudinal section of a needle from a potassium-deficient tree frozen to -18° C on February 7. Damage index: mesophyll, 2.00; transfusion tissue, 2.00. The abaxial mesophyll was severely injured under the endodermis. 80x.

Figure 40. Upper and lower surface of needles from a potassium-deficient tree frozen to -18° C on November 3. Lower needle of each pair is unfrozen. Damage index: mesophyll, 2.50; transfusion tissue, 3.00. A dark midrib on the lower surface was characteristic of severe injury. 6x.

Effect of Potassium Deficiency and Growth

Twigs from potassium-deficient trees remained succulent longer and developed freezing resistance at a later date than twigs from nondeficient trees. These observations prompted in part study of the relation between potassium content of the current season's foliage, growth response after winter dormancy as measured by date of flushing, and freezing injury during autumn and spring.

Results and Discussion

Average potassium content of the current season's foliage for potassium-deficient and nondeficient trees increased gradually in late summer and early autumn, declined sharply to a minimum in early winter, and increased slightly in late winter (Figure 41). Potassium content of the current season's foliage during January showed a range from 0.12 to 0.34 percent for trees from the potassium-deficient area (Table I, Appendix) and a range from 0.37 to 0.71 percent for trees from the nondeficient area (Table II, Appendix). Foliage analysis indicated that none of the other essential elements examined (nitrogen, phosphorous, calcium, magnesium, sulfur, copper, iron, manganese, and zinc) were deficient in the potassium-deficient trees.

Vegetative apices of potassium-deficient trees did not grow until several weeks after growth commenced in vegetative apices of nondeficient trees brought into the laboratory in midwinter and kept there at 25° C. This observation suggested that lack of potassium may inhibit growth and delay loss of freezing resistance.

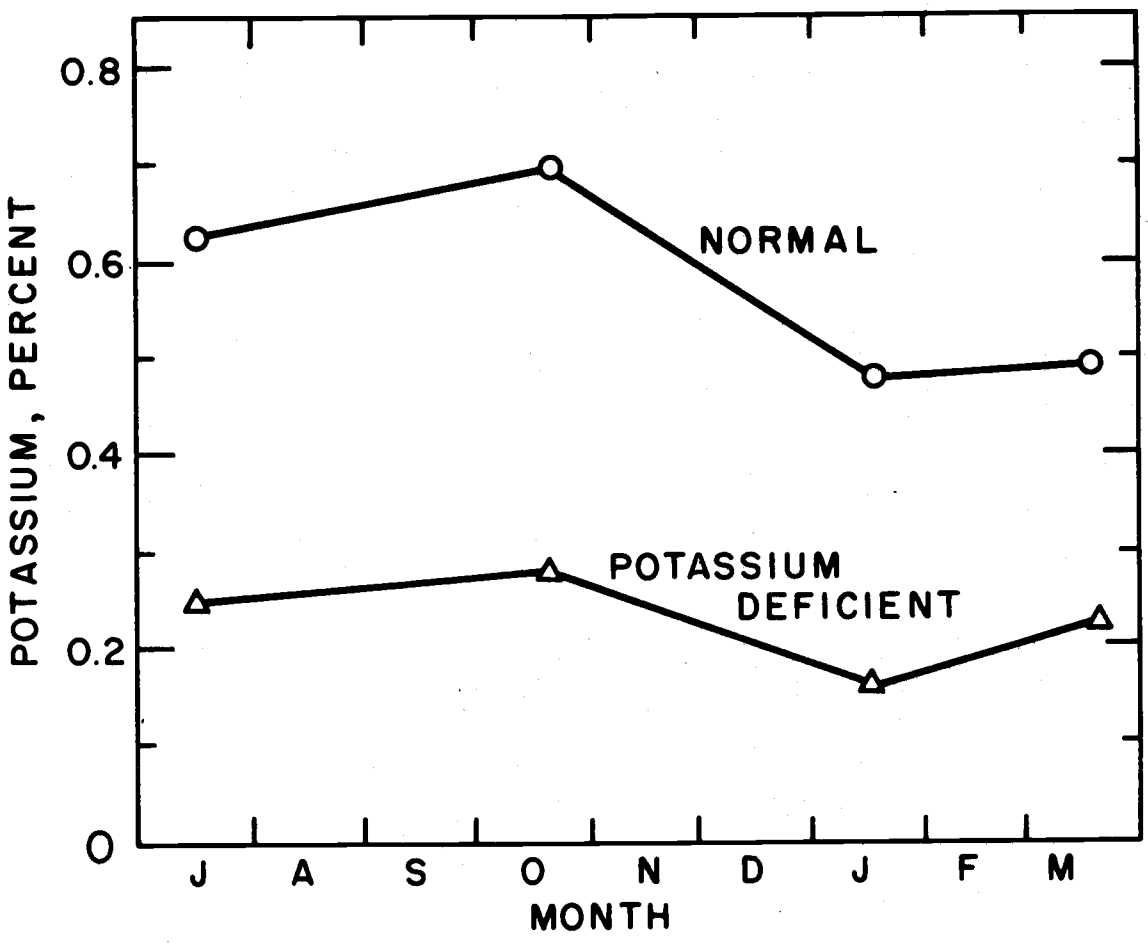

Figure 41. Seasonal change in potassium content of the foliage of potassium-deficient and nondeficient Douglas-fir.

Freezing resistance of the first set of sample trees from the potassium-deficient area could not be compared to the freezing resistance of trees from the nondeficient area with confidence, however, because seed source of the potassium-deficient trees was doubtful. Relation between freezing resistance, potassium content, and date of flushing was determined by separate regression analysis for trees within the potassium-deficient area and for trees within the nondeficient area. Data chosen for analysis were from the periods of development and loss of hardiness during which injury was highly variable (Tables IV and V). Regression analysis had to be restricted to trials with test temperatures that provided maximal distribution of freezing resistance from little or no injury for the most resistant trees to severe injury or death for the most susceptible trees. Difference in resistance between tissues also limited the number of trials for which regression analysis was applicable.

Data from Tables IV and V indicate that trees with late bud burst developed freezing resistance in the autumn and lost freezing resistance in the spring more slowly than trees that flushed early. Number of days to bud burst after March 31 was also more closely related to development and loss of freezing resistance than potassium content for all tissues examined except transfusion tissue of the needle.

The lower the potassium content of the foliage the later was the date of bud burst for trees from the second selection in the potassium-deficient area (Figure 42, Table V and Table I, Appendix).

Table IV. Correlation between days to bud burst (BB) after March 31, percent potassium content [1] of the foliage (K) and freezing injury (Di) for tissues of the stem, needle and lateral buds of nondeficient trees.

Tissue	Test Temp.	Date of Freezing Test	Autumn[2] Average BB	K	Di	Correlation BB vs. K	BB vs. Di	K vs. Di
Stem								
Phloem-cambium	-18° C	11-3	22	.50	1.98	.45	.39	-.08
Cortex	-7.5° C	8-22	22	.50	1.86	.45	-.29	.07
Pith	-18.5° C	11-20	22	.50	1.65	.45	.61*	-.08
Needle								
Transfusion tissue	-18.5° C	11-20	22	.50	2.08	.45	.29	.19
			Spring[2]					
Stem								
Phloem-cambium	-14° C	3-19	23	.51	2.01	.31	-.64**	-.42
Cortex	-14° C	3-26	23	.51	2.18	.31	-.71**	-.38
Pith	-10° C	4-11	23	.51	2.24	.31	-.11	-.23
Needle								
Transfusion tissue	-14° C	3-19	23	.51	2.03	.31	-.26	-.06
Buds								
Vegetative apices	-10° C	4-1	23	.51	1.89	.31	-.68**	-.41

*Correlation is significant at the .95 probability level.
**Correlation is significant at the .99 probability level (Table VII, p. 63, Fisher and Yates, 1963).
1/Based on January potassium contents.
2/Autumn determinations are based on trees from the first selection; spring determinations are based on trees from the first and second selections.

Table V. Correlation between days to bud burst (BB) after March 31, percent potassium content [1] of the foliage (K) and freezing injury (Di) for tissues of the stem, needle and lateral buds of potassium-deficient trees.

Tissue	Test Temp.	Date of Freezing Test	Average BB	Average K	Average Di	Correlation BB vs. K	Correlation BB vs. Di	Correlation K vs. Di
Autumn [2]								
Stem								
Phloem-cambium	-18° C	11-3	26	.23	2.21	-.16	.32	.41
Cortex	-18° C	11-3	26	.23	1.40	-.16	.59*	.12
Pith	-7.5° C	8-22	26	.23	2.44	-.16	.78**	-.62*
Needle								
Transfusion tissue	-18.5° C	11-22	26	.23	1.71	-.16	.20	.16
Buds								
Vegetative apices	-18° C	11-3	26	.23	1.78	-.16	.26	.00
Spring [2]								
Stem								
Phloem-cambium	-6° C	4-27	26	.27	2.31	-.41*	-.69**	.33
Cortex	-10° C	4-11	24	.28	2.00	-.33	-.72**	.26
Pith	-18° C	3-17	26	.29	1.78	-.62**	-.50*	.34
Needle								
Transfusion tissue	-14° C	3-26	26	.27	2.15	-.41*	-.24	.68**
Buds								
Vegetative apices	-10° C	4-11	24	.28	1.71	-.33	-.26	-.28

*Correlation is significant at the .95 probability level.
**Correlation is significant at the .99 probability level (Table VII, p. 63, Fisher and Yates, 1963).
1/Based on March potassium contents.
2/Autumn determinations are based on trees from the first selection; spring determinations are based on trees from the first and second selections.

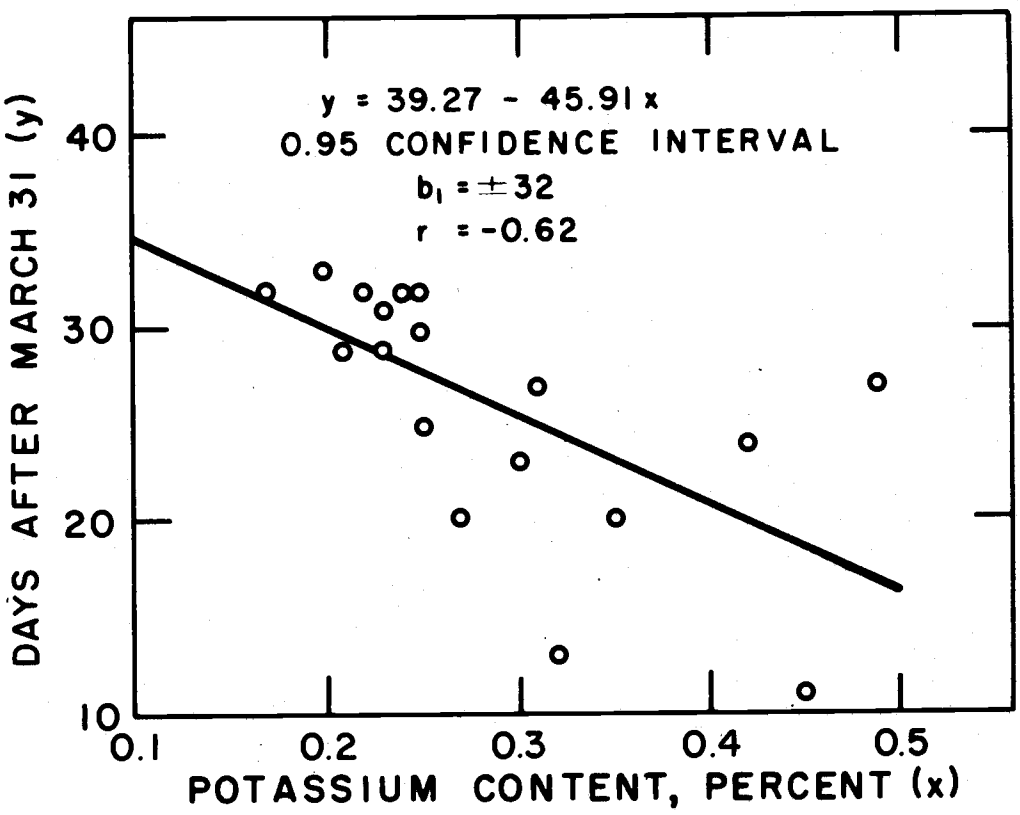

Figure 42. Effect of potassium content of the foliage on bud burst. Slope of the regression is statistically significant ($P < 0.01$) from 0.

This relationship was not apparent for trees from the potassium-nondeficient area and for trees from the original selection in the potassium-deficient area (Tables IV and V). The reasons were not apparent why trees from the original selection in the deficient area did not show an effect of potassium nutrition on date of bud burst. Most trees were selected from the center of the deficient area but range in potassium contents may have been inadequate to show the effects of deficiency. However, observation of the 18 trees from the second selection suggested a relation between potassium deficiency and days to bud burst after March 31 (Figure 42).

Error in estimating injury, slow rate of flushing after initial bud burst (Table VI), and variation in freezing resistance between twigs from north to south aspects of the same tree (Table VII), accounted in part for unexplained variation in regression of date of bud burst as a measure of growth response and potassium content of the current season's foliage against injury. Twigs subjected to freezing tests had not been selected on the basis of bud development or aspect.

Analysis of variance for the data summarized in Table VII also revealed that difference in injury between trees was significant at the .99 probability level for tissues of the stem but not for vegetative apices of lateral buds. Unlike other tissues in the twig, cold resistance of vegetative apices depended on their ability to remain subcooled at subfreezing temperatures because they could not tolerate formation of ice. This factor may explain the lack of significant correlation between date of bud burst and injury to the vegetative

Table VI. Range, median and average days for bud burst (BB) of potassium-deficient and nondeficient trees after March 31.

	Normal Trees			Potassium-deficient Trees		
	South Aspect	North Aspect	Difference	South Aspect	North Aspect	Difference
Range in BB	27	28		29	28	
Average BB	23	28	5	26	36	10
Median BB	24	29	5	27	38	11
Trees fully flushed on May 11, percent		82			13	

Table VII. Average injury for tissues of three twigs each from north and south aspects of the same tree 13 days after exposure to -6° C on May 2 and correlation coefficient between bud enlargement and injury.

Tissue	South Aspect	North Aspect	Difference	r
Stem				
Phloem-cambium	2.58	2.01	.57**	.79**
Cortex	1.84	1.30	.54**	.66**
Pith	1.79	1.27	.52**	.63**
Needle				
Transfusion tissue	1.36	1.25	.09	.30*
Bud				
Vegetative apices	1.77	1.18	.56*	.55**

*Significant at the .95 probability level.
**Significant at the .99 probability level. (Table VII, p. 63, Fisher and Yates, 1963).

apices in Table V. Freezing resistance of the transfusion tissue was unrelated to exposure of the twig and was not strongly correlated with bud development as a measure of growth response (Table VII). These findings support the assumption that date of bud burst after March 31 and injury to transfusion tissue were independent (Tables IV and V) because twigs were selected from all aspects of the tree for freezing tests.

The decrease in days to bud burst with increasing potassium content of the current season's foliage (Figure 42) and the positive correlation between days to bud burst and injury from freezing in the autumn (Figure 43) agreed with reports that high potassium nutrition enhances early development of frost resistance. Negative correlations between days to bud burst and injury from freezing in the spring (Figure 44) indicated that potassium deficiency delayed onset of growth processes and loss of resistance to freezing injury. Absence of measurable variation in freezing injury between trees after development of freezing resistance suggested that ultimate frost resistance was not related to date of flushing although potassium deficiency may have reduced the tree's capacity to develop maximal resistance (Table II).

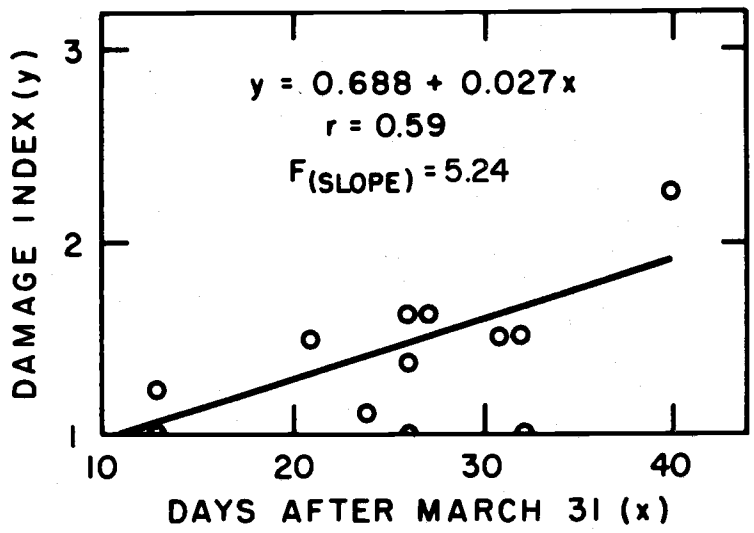

Figure 43. Relation between date of bud burst and injury to the cortex of potassium-deficient twigs frozen to -18° C on November 11. Slope of the regression is statistically significant ($P < 0.01$) from 0.

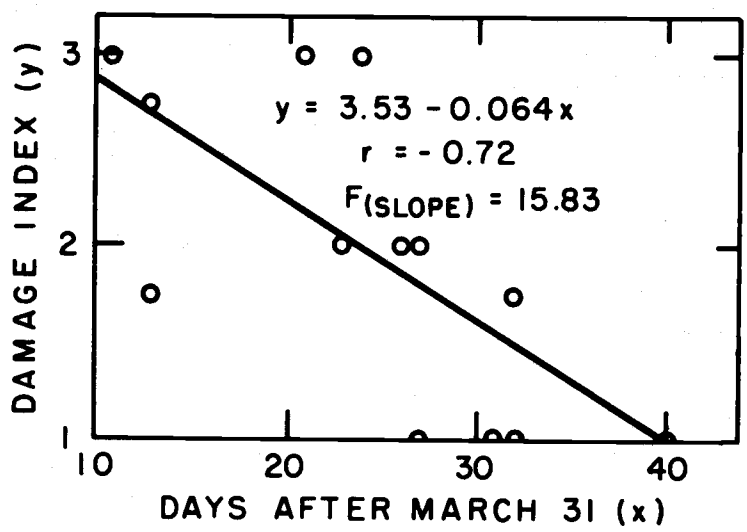

Figure 44. Relation between date of bud burst and injury to the cortex of potassium-deficient twigs frozen to -10° C on April 11. Slope of the regression is statistically significant ($P < 0.01$) from 0.

INCREASED FREEZING RESISTANCE OF DOUGLAS-FIR IN RESPONSE TO LOW TEMPERATURES

Development of freezing resistance appears to follow an endogenous rhythm in plants capable of acquiring hardiness. The process of hardening begins in midsummer with cessation of growth processes and terminates in early winter in response to subfreezing temperatures and often enables plants to endure temperatures far below those of the most severe habitats. Light is essential during early stages of hardening for accumulation of photosynthetic reserves after growth ceases. Short photoperiods initiate synthesis of hardiness promoting substances and induce dormancy. Most researchers, however, agree that low temperature influences development of cold tolerance more than any other environmental factor. While low temperatures above 0° C with short days reduce growth and initiate the first stage of hardiness, maximal freezing resistance develops in response to subfreezing temperatures. Van den Driessche (1969) reported that seedlings of Douglas-fir developed freezing resistance in response to low night temperatures even when day temperatures were above 20° C. However, the influence of subfreezing temperatures on the ultimate hardiness of Douglas-fir is still unknown. For this reason, the effect of temperatures below the current season's minimum (-5° C) was investigated to determine if Douglas-fir is capable of developing freezing resistance in excess of that induced by its natural environment.

Procedure

Four cuttings per tree were collected from 6 nondeficient and 18 potassium-deficient trees on December 31. One cutting per tree was frozen immediately to -26° C, two cuttings per tree were placed in a cold storage room at -9° C and one cutting per tree was placed in a second cold storage room at 2° C. The severed end of each cutting was sealed with paraffin and all cuttings were enclosed in plastic bags to prevent loss of moisture. All cuttings were hardened for three days in darkness and then frozen to -26° C. My previous tests revealed that periods of hardening longer than four days at -9° C caused injury to the transfusion and mesophyll tissues of the foliage. Injury was evaluated about two weeks after freezing according to the technique described under Methods.

A second trial was repeated on February 22 after the trees had lost some of their freezing resistance. Modifications of the above procedure included an additional hardening treatment at 2° C with severed ends of the cuttings in water, a hardening period of four instead of three days and a test temperature of -18° C instead of -26° C. The number of trees sampled from the potassium-deficient area was also reduced from 18 to 11. All results were tested by analysis of the variance and significant differences between means were determined by Duncan's new multiple range test (Harter, 1960).

Results and Discussion

Transfusion and vegetative apices of excised twigs from nondeficient trees subjected to -9° C for three days in January showed greater resistance to freezing injury than twigs with no treatment and twigs with a 2° C pretreatment when frozen to -26° C (Table VIII). Hardiness of foliage from potassium-deficient trees increased as a result of the -9° C pretreatment (Table IX).

The phloem-cambium region and pith of the stem in potassium-deficient trees showed increased freezing resistance following both the 9° C and 2° C hardening treatments (Table IX). Test temperatures of -26° C did not produce enough injury to separate the effects of hardening on mesophyll and cortex tissues of nondeficient trees and the cortex of potassium-deficient trees.

None of the tissues exposed to -9° C for four days in late February increased in hardiness and the mesophyll and cortex lost freezing resistance (Tables X and XI). Hardiness of tissues from twigs maintained at 2° C in water was not significantly different from those kept at -9° C, except for terminal apices of potassium-deficient trees. Freezing resistance of the phloem-cambium region, pith and vascular tissue of the bud trace increased after four days at 2° C when severed ends of the twigs were sealed with paraffin. Freezing resistance of all tissues from potassium-nondeficient twigs kept under moisture stress at 2° C was significantly greater than tissues of twigs maintained in water at 2° C, except for vegetative apices and tissues of the stem enclosed by coriaceous bud scales.

Table VIII. Effect of low temperature treatments on the freezing resistance of twigs from potassium-nondeficient Douglas-fir collected on December 31 and frozen to -26° C. Data represent means of six samples.

Hardening Treatment	Damage Index [1/] Needle: Transfusion Tissue	Needle: Mesophyll	Stem: Cortex	Stem: Phloem-cambium	Stem: Pith	Terminal and Lateral Buds: Stem Enclosed by Coriaceous Scales	Terminal and Lateral Buds: Vegetative Apices	Terminal and Lateral Buds: Vascular Tissue of the Trace
-9° C	1.33[b]	1.00	1.00	1.63	1.58	1.06[a]	1.17[a]	1.75
2° C	1.96	1.33	1.00	1.83	1.75	1.31[ab]	3.00	2.00
Untreated	1.88	1.12	1.00	1.71	1.58	1.47[b]	3.00	2.00
Control at -9° C	1.00[a]	1.00	1.00	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]

[1/] No significant difference among means is indicated for those treatments sharing a common letter; statistical significance ($P < 0.05$) is indicated for treatments not sharing a common letter.

Table IX. Effect of low temperature treatments on the freezing resistance of twigs from potassium-deficient Douglas-fir collected on December 31 and frozen to -26° C. Data represent means of eighteen samples.

	Damage Index [1]							
	Needle		Stem			Terminal and Lateral Buds		
Hardening Treatment	Transfusion Tissue	Mesophyll	Cortex	Phloem-cambium	Pith	Stem Enclosed by Coriaceous Scales	Vegetative Apices	Vascular Tissue of the Trace
-9° C	1.56[b]	1.45[b]	1.08	1.58[b]	1.30[b]	1.06[a]	1.06[a]	1.76[a]
2° C	1.96	1.69	1.11	1.69[b]	1.58[c]	1.45	3.00	1.92
Untreated	2.15	1.76	1.00	1.94	1.89	1.52	3.00	1.97
Control at -9° C	1.00[a]	1.03[a]	1.00	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]

[1] No significant difference among means is indicated for those treatments sharing a common letter; statistical significance ($P < 0.05$) is indicated for treatments not sharing a common letter.

Table X. Effect of low temperature treatments on the freezing resistance of twigs from potassium-nondeficient Douglas-fir collected on February 22 and frozen to -18° C. Data represent means of six samples.

	Damage Index [1]								
	Needle		Stem			Terminal and Lateral Buds			
Hardening Treatment	Transfusion Tissue	Mesophyll	Cortex	Phloem-cambium	Pith	Stem Enclosed by Coriaceous Scales	Vegetative Apices Term.	Vegetative Apices Lat.	Vascular Tissue of the Trace
-9° C	1.79^{bc}	1.83	1.42	1.88	1.67^{bc}	1.03	1.00	1.00	1.75
2° C dry	1.29^{ab}	1.21^{a}	1.00^{a}	1.38^{b}	1.25^{ab}	1.00	1.00	1.11	1.42^{b}
2° C wet	2.25^{c}	1.92	1.50	2.04	1.83^{c}	1.19	1.67	1.00	1.92
Untreated	1.83^{bc}	1.21^{a}	1.00^{a}	1.79	1.54^{bc}	1.06	1.67	1.00	1.92
Control at -9° C	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00	1.00	1.00	1.00^{a}

[1] No significant difference among means is indicated for those treatments sharing a common letter; statistical significance ($P < 0.05$) is indicated for treatments not sharing a common letter.

Table XI. Effect of low temperature treatments on the freezing resistance of twigs from potassium-deficient Douglas-fir collected on February 22 and frozen to -18° C. Data represent means of eleven samples.

	Damage Index [1]								
	Needle		Stem			Terminal and Lateral Buds			
Hardening Treatment	Transfusion Tissue	Mesophyll	Cortex	Phloem-cambium	Pith	Stem Enclosed by Coriaceous Scales	Vegetative Apices Term.	Vegetative Apices Lat.	Vascular Tissue of the Trace
-9° C	2.09^{cd}	1.81	1.20	1.81	1.53^{bc}	1.05	1.00	1.00	1.70
2° C dry	1.81^{bc}	1.63	1.18	1.50^{b}	1.32^{b}	1.00	1.00	1.00	1.45^{b}
2° C wet	2.22^{d}	1.88	1.23	1.93	1.68^{c}	1.10	1.55^{a}	1.09	1.77
Untreated	1.68^{b}	1.30^{a}	1.05	1.84	1.68^{c}	1.02	1.00	1.06	1.77
Control at -9° C	1.18^{a}	1.13^{a}	1.00	1.11^{a}	1.00^{a}	1.00	1.00	1.00	1.02^{a}

[1] No significant difference among means is indicated for those treatments sharing a common letter; statistical significance ($P < 0.05$) is indicated for treatments not sharing a common letter.

Test temperatures of -18° C in February, as -26° C in January, did not cause enough injury to separate the effects of hardening on the cortex and vegetative apices. Loss of freezing resistance of mesophyll tissue of potassium-deficient twigs held at 2° C without water was the most apparent difference between potassium-deficient and potassium-nondeficient trees in February. Potassium-deficient twigs held as controls were also injured by the -9° C hardening treatment. Tissues of both potassium-deficient twigs and nondeficient twigs were slightly injured after three or four days in darkness at -9° C even during the period of greatest freezing resistance.

Failure of Douglas-fir to develop freezing resistance in darkness at -9° C during late winter may have been associated with initiation of growth processes that coincided with loss of hardiness. Increase in freezing resistance of those twigs without water, and loss of resistance of those twigs held in water at 2° C, suggested that moisture stress was an important factor in freezing resistance at this time.

The increase in freezing resistance of vegetative apices, tissues of the foliage, and stem after hardening at -9° C in December indicated that Douglas-fir was capable of developing greater cold tolerance when exposed to temperatures that impart maximal freezing resistance in hardy species. Subfreezing temperatures in December may have induced greater freezing resistance to tissues of the foliage than tissue of the stem because the mesophyll and transfusion tissue did not reach maximal freezing resistance in the outside environment until mid-January (Figures 12 and 13). The striking increase in freezing resistance of vegetative apices after exposure to -9° C in December

(Tables VIII and IX) suggested that subfreezing temperatures must have increased the ability of these tissues to avoid ice formation because death was instantaneous and complete at the moment of freezing (Table III).

A MECHANISM OF INCREASED FREEZING RESISTANCE OF THE VEGETATIVE APEX

My previous work showed that vegetative apices of Douglas-fir hardened at subfreezing temperatures although they could not tolerate ice and were killed at the moment of ice formation. Severe water soaking of the pith, scales and cortex under the crown of hardened buds without injury after freezing and thawing suggested that these tissues probably accumulated large quantities of ice from water in the apex. Apparently, cells of the apex reabsorbed water from vascular tissue after thawing and left intercellular spaces and cell walls of the pith, scales and cortex saturated with water from ice that had accumulated in these tissues.

Work with unicellular organisms and hardened tissue of higher plants with intercellular space has established that ice continues to form after extracellular water crystallizes because intracellular water diffuses out of cells in response to reduced vapor pressure in the vicinity of the ice crystals (Mazur, 1969 and 1970). Mazur (1969) concluded that slow cooling velocities and high permeability of cell membranes to water prevent formation of lethal intracellular ice but that they do not prevent injuries caused by dehydration of the protoplasm. He calculated that intracellular water of yeast cells is incapable of subcooling at freezing rates less than 10° C per minute. Samygin and Bliadze (1969) reported that only extracellular ice forms in tissues of lemon and tea plants at freezing rates less than 2° C per minute in the absence of subcooling but Samygin

and Matveeva (1963) observed intracellular ice in cabbage cooled at 10° C per hour.

These observations suggest that ice in tissues of the bud with extracellular space could dehydrate the vegetative apex and reduce the temperature of ice nucleation in water that remains associated with the protoplasm and undeveloped cell walls. I conducted several experiments to test this hypothesis.

Procedure

Twelve cuttings from a Douglas-fir growing near the Forest Research Laboratory at Corvallis were hardened at -9° C on January 23 to determine if ice in the pith of buds reduces the moisture content and increases the freezing resistance of the vegetative apex. An additional 12 cuttings from the same tree were hardened at 2° C. The severed end of each cutting was sealed with paraffin to prevent excessive loss of moisture. After four-and-one-half days six cuttings from each treatment, and six unhardened cuttings were cooled at the rate of 9° C per hour to -20° C for 20 minutes and warmed 5° C per minute to room temperature. The number of surviving apices were determined after one week at room temperature.

The moisture content of the pith directly under the crown and the vegetative apex including the crown was analyzed for the remaining six cuttings. The terminal apex and two lateral apices of each stem were recovered by removing the bud scales and cutting transversely through tissues at the base of the crown. Three millimeters of the adjacent pith of each bud was also recovered by removing the

vascular tissue and cortex. Both samples were weighed to the nearest 0.1 mg in one dram vials within ten minutes after recovery to determine fresh weights. The vegetative apices were dried at 70° C for one hour, and the pith for 30 minutes, and reweighed. Results were tested by analysis of variance and all means were compared by Duncan's new multiple range test (Harter, 1960).

Two additional experiments were conducted on February 5 and 18 to determine the rate of dehydration of the vegetative apex, hydration of the underlying pith and increase in freezing resistance of the vegetative apex while hardening at subfreezing temperatures. Cuttings from the same tree were hardened in darkness at 2° and -9° C. Cuttings for one treatment at 2° C were placed in water. The severed ends of all other cuttings were sealed with paraffin. Two cuttings were removed periodically (Figure 45) beginning at five hours after initiation of each treatment to determine the moisture content of the vegetative apex, pith and temperature of ice nucleation in the apex. Subcooling points were determined by inserting a copper-constantan thermocouple of 30-gauge wire into the pith at the base of the terminal bud. Change in temperature of the twig was recorded continuously during freezing and thawing to about -5° C. Test tubes containing the twigs were submersed in precooled ethylene glycol of the freezing chamber to provide a freezing rate of about 1° C per minute. Rate of freezing was highly variable although the bath temperature remained constant because formation of ice was irregular and large twigs lost heat more slowly than small twigs.

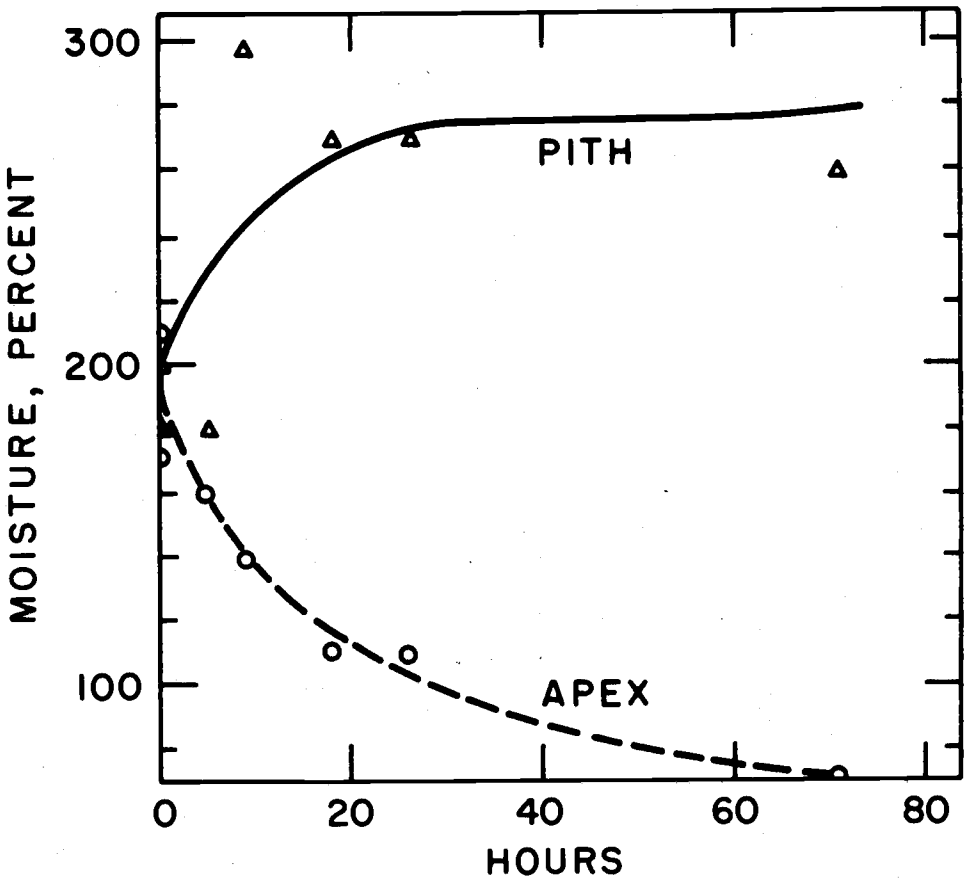

Figure 45. Rate of diffusion of water from the vegetative apex to the pith at -9° C.

During the final experiment on February 18, cuttings from two trees were hardened in the ethylene glycol bath of the freezing chamber at -3° C. Severed ends of cuttings were either submerged in 3 to 5 mm of water or sealed in paraffin to prevent moisture loss. Previous tests established that ice forms in tissues of twigs in water within 20 minutes at -3° C while twigs in dry test tubes remain subcooled longer than four days. Cuttings were removed periodically after two-and-one-half hours (Figure 46) and moisture content of the vegetative apex and pith was determined as above.

Results and Discussion

Results of the first experiment are summarized in Table XII. Water content of the vegetative apex decreased while water content of the pith increased during hardening at -9° C. Slow dehydration at 2° C, however, reduced water content uniformly in both the pith and vegetative apex but not to the same extent as did ice formation in the pith. Reduction in water content of the vegetative apex by dehydration at 2° C, as well as by ice formation, appeared to increase the freezing resistance of the vegetative apex. Vegetative apices failed to harden at 2° C when they remained fully hydrated (Figures 47 and 48). These findings suggested that diffusion of freezable water from the protoplasm to sites of extracellular ice, and not low temperature, was the direct cause of hardening at subfreezing temperatures. Low temperatures, however, have been shown to determine the rate and extent of water loss from the protoplasm

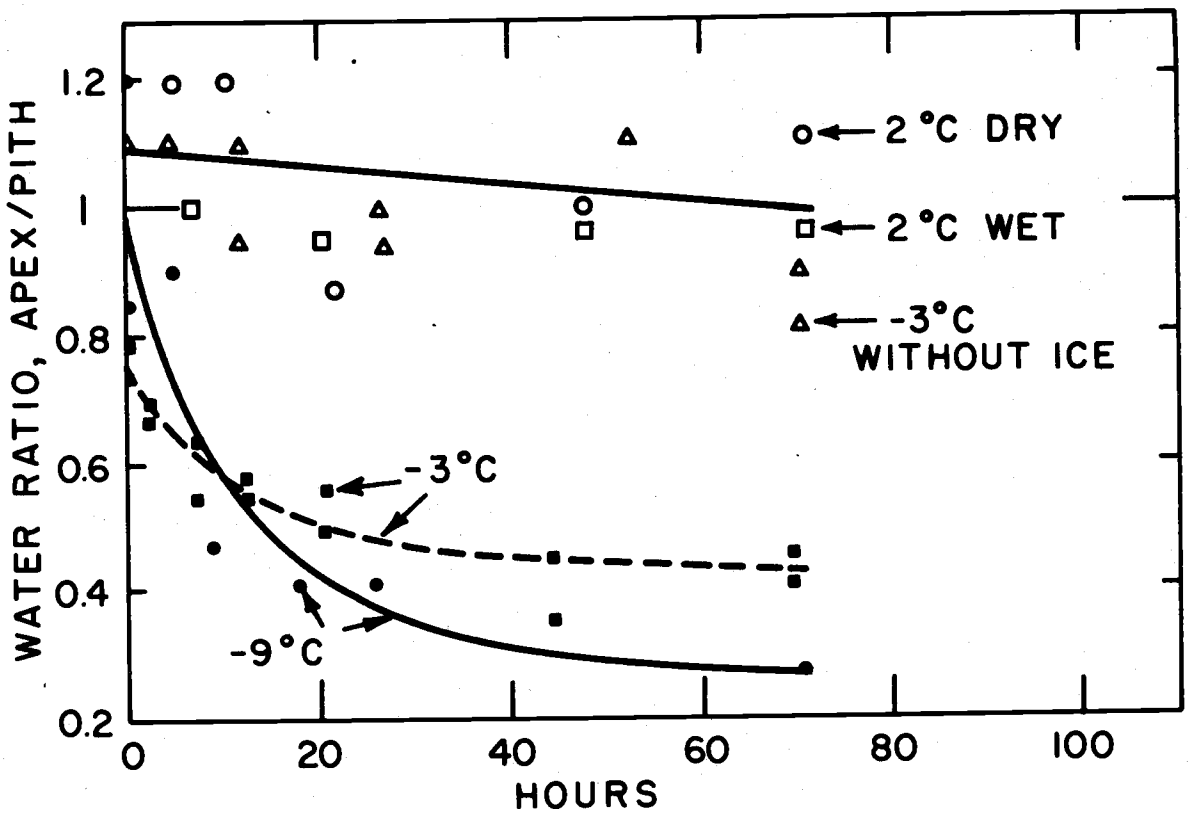

Figure 46. Effect of hours of hardening at -9° C, -3° C with and without ice and 2° C with and without water on the ratio of water contents of the apex to pith.

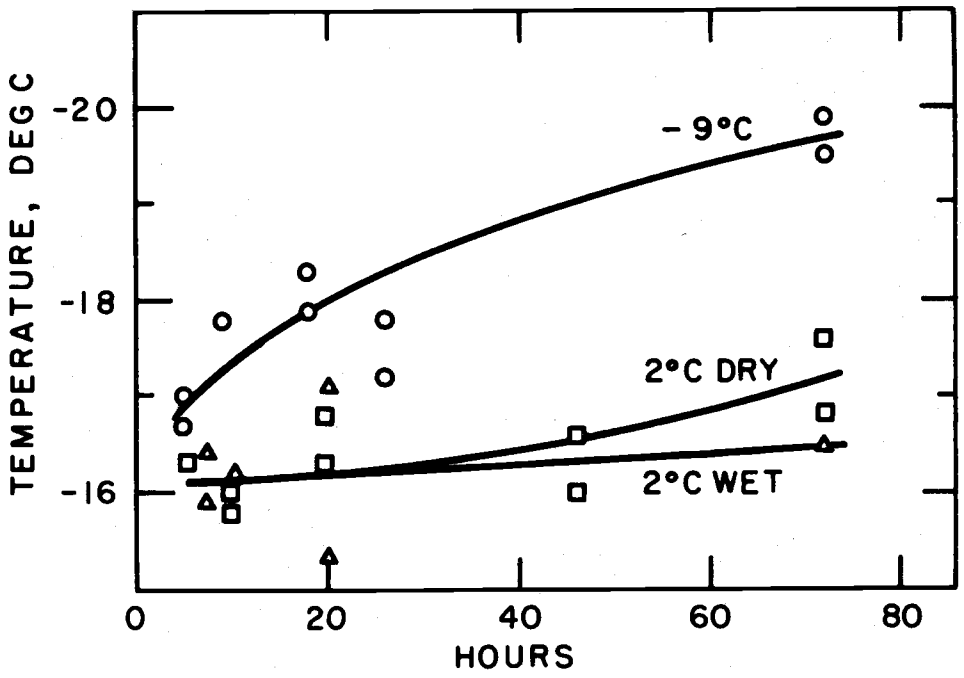

Figure 47. Effect of hours of hardening at -9° C, 2° C dry and 2° C wet on the subcooling point of the vegetative apex.

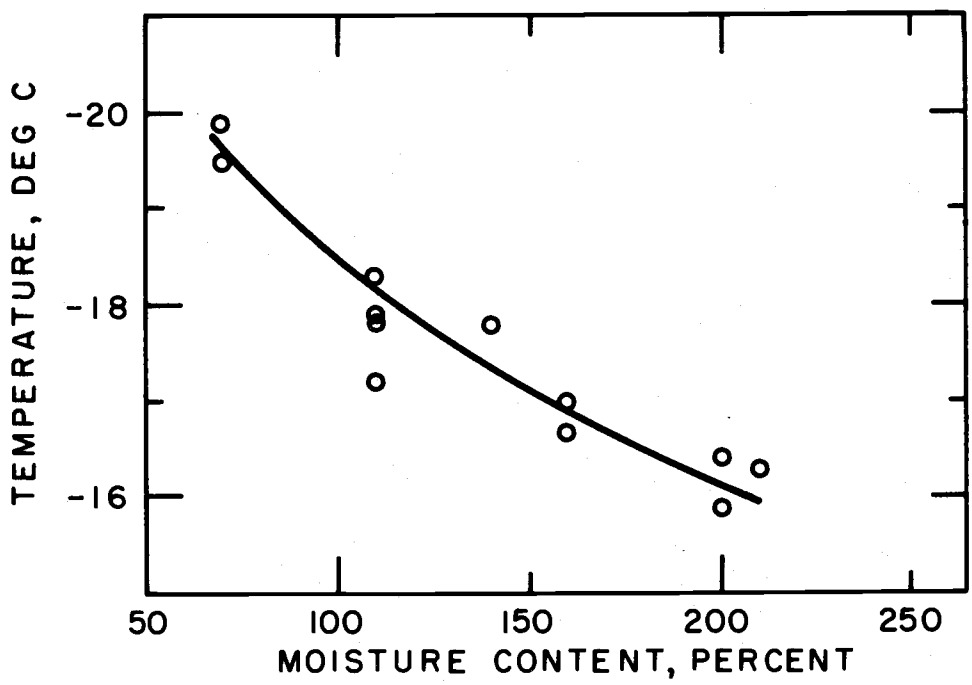

Figure 48. Relation between moisture content and subcooling point of the vegetative apex.

(Sakai, 1965a and 1965b; Tumanov and Krasavtsev, 1966a and 1966b; Mazur, 1969). Low temperatures may change macromolecular conformation and water structure, reduce energy of hydrophobic bonds which in turn reduce water under the influence of macromolecules (Brandts, 1967) increase permeability of the protoplasm to water and reduce the water retaining capacity of the protoplasm.

Table XII. Average moisture content of the vegetative apex and pith of the buds of Douglas-fir and survival of the vegetative apex frozen to -20° C after four-and-one-half days at low temperature.

Treatment	Percent Moisture [1/] Dry Weight Basis		Percent Survival	
	Apex	Pith	Terminal Bud	Lateral Bud
Untreated	214 ± 16	202 ± 18	0	30
-9° C	60 ± 8	260 ± 34	100	100
2° C	122 ± 10	127 ± 22	100	95

[1/] Includes .95 confidence intervals. Moisture content of all treatments are significantly different at the .99 probability level.

Figures 45 and 46 show that water diffused from the vegetative apex into the pith of the bud at -9° C. Water content of the apex decreased from about 200 to 70 percent and water content of the pith increased from about 200 to 270 percent on a dry weight basis after three days at -9° C (Figure 45). Small sample size and evaporative loss introduced considerable error in determining moisture contents of the pith. Water content of the vegetative apex was reduced more than 50 percent after 24 hours at -9° C, nevertheless. Several recent studies have shown even more rapid dehydration of

tissues at subfreezing temperatures. Krasavtsev (1969a) found that dehydration of cells from hardy branches of birch and poplar was complete in less than one day at -5° C. Calorimetric measurements have also shown that release of heat on fusion of water in one-year-old branches of hardened birch was complete in six to eight hours after each successive 10° C reduction in temperature to -60° C (Krasavtsev, 1969b).

Incipient flow of intracellular water from the cell after formation of extracellular ice apparently results from a vapor pressure gradient because ice has a lower vapor pressure than subcooled water at the same temperature, and the ratio of intracellular to extracellular vapor pressure increases with decreasing temperature if water does not flow out of the cell and the cytoplasm obeys Raoult's law (Mazur, 1969 and 1970). The rate of water movement out of a cell depends on permeability of the protoplasm to water, area of the cell surface and difference in chemical potentials of the intracellular and extracellular water. The cell will lose water even before the extracellular osmotic pressure exceeds the intracellular because of the vapor pressure gradient and cell wall pressure on the protoplasm.

The flow of water from cells of the vegetative apex was more rapid at -9° C than at -3° C (Figure 46) because of a greater vapor pressure gradient. Such factors as size, number of cells, geometry, surface area exposed to dehydration, permeability of cell membranes and difference in chemical potential of intercellular water may have accounted for the slow rate of water loss from the vegetative apex

at subfreezing temperatures when compared with rapid loss of water from unicellular organisms. Calculations of Mazur (1969, 1970) showed that the moisture content of yeast cells was reduced 50 percent in less than five minutes when frozen at 1° C per minute. However, calculated rates of intracellular water loss based on internal and external osmotic pressure and vapor pressure gradient imply that the protoplasm is an aqueous solution of nonelectrolytes surrounded by a membrane that is permeable only to water. Structural macromolecules of the protoplasm and cell walls may also contribute to the structure and chemical potential of water in plant tissues (Boyer, 1969).

In my experiments, subcooling temperature of the vegetative apices frozen at a rate of 1° C per minute decreased from about -16° C to -20° C after three days as the buds hardened at -9° C (Figure 47). This increase in freezing resistance resulted from reduction in moisture content of the apices (Figure 48). Several lateral buds survived temperatures lower than -20° C after three days at -9° C, however. Additional dehydration of vegetative apices either at temperatures lower than -9° C or by longer periods of exposure at -9° C may enable buds of Douglas-fir to survive temperatures of liquid nitrogen. The subcooling point and moisture content of the vegetative apex for both treatments at 2° C did not change during the experiment (Figures 46 and 47).

The increase in subcooling ability of the vegetative apex as water decreased, undoubtedly accounted for increases in cold resistance of peach fruit buds observed after winter frosts by Meader and

and Blake (1943) and Chaplin (1948). Tumanov and Krasavtsev (1959) suggested that low temperatures and dehydration of plant cells reorganize the protoplasm by decreasing molecular motion and intermolecular distance. The new ordered structure increases permeability and resistance of the protoplasm to mechanical deformation and injurious effects of dehydration. Furthermore, Krasavtsev (1969b) suggested that dehydration changes the structure of unstable macromolecules and reduces their water retaining capacity. The water containing capacity is reversible before injury to tissues of woody plants at -30° C to -40° C but is irreversible after death at -50° C to -60° C. Many plants, the winter cereals in particular, do not develop sufficient water retaining capacity to protect intracellular water from freezing during very severe frosts and high freezing resistance develops only after the protoplasm is dehydrated (Tumanov, Krasavtsev and Trunova, 1969).

Dehydration of the vegetative apex of Douglas-fir in my experiments, however, reduced the temperature of nucleation of ice and delayed freezing of water that remained under the influence of macromolecules of the cell wall and protoplasm (Figure 48). Cell walls of the crown were especially thick and may have inhibited inoculation of the vegetative apex with ice from underlying tissues of the bud and stem. When freezing occurred, ice may have originated at nucleating sites associated with cell walls of the vegetative apex and then in the protoplasm, because two subcooling points characteristic of plant tissues were evident in detached apices. Most researchers believe that the first subcooling point represents freezing of

extracellular water but interpretation of the second subcooling point varies widely. Cells of the vegetative apex of many plants can survive -196° C and lower temperatures after dehydration if water, that remains associated with the protoplasm and cell walls, is capable of vitrification. Sakai (1965a and 1965b) demonstrated that hardened buds of numerous woody plants can survive rapid freezing in liquid nitrogen providing all freezable water is removed from the protoplasm. Effect of prefreezing temperatures that removed intracellular water depended on species and hardiness and ranged from -15° to -30° C.

A gradual reduction in size of the exotherm associated with death of the vegetative apex of Douglas-fir was observed in my expermments as water content declined during hardening at -9° C. This observation indicated that death was not directly caused by freezing of water that was easily removed from the protoplasm during dehydration because injury was abrupt and complete regardless of the size of the exotherm. Thus death was apparently caused by freezing of water that was under the direct influence of protoplasmic constituents and was essential for stabilizing the vital conformation of freezing sensitive macromolecules.

Early investigations of the freezing resistance of fruit buds of peach support the hypothesis that formation of ice in tissues with intercellular space dehydrates the vegetative apex and promotes subcooling of water associated with macromolecules of the protoplasm and cell walls. Johnston (1922) found that peach buds subcooled in winter before death at -6° to -8° C. Freezing resistance of the fruit

bud was directly related to moisture content and declined as moisture content increased with mean daily temperature after January 1 (Johnston, 1923). Dorsey (1934) also studied the freezing resistance and ice formation in the fruit bud of peach. He observed that ice in the pith core and large cells of the bud scales dehydrated rudimentary flower parts and increased their freezing resistance. He concluded that removal of water from meristematic tissues by freezing has an important bearing on the cold resistance of the fruit bud.

SEASONAL CHANGE IN RESISTANCE TO INJURY FROM ICE FORMATION

Numerous studies have been conducted to show that hardy plants and even nonhardy unicellular organisms can survive extremely low temperatures if formation and growth of intracellular ice crystals are prevented during freezing and thawing (Levitt, 1956; Tumanov and Krasavtsev, 1966a and 1966b; Sakai and Otsuka, 1967; Sakai and Yoshida, 1967; Krasavtsev, 1967; Sakai, Otsuka and Yoshida, 1968 and Mazur, 1969). In addition, many microorganisms (Mazur, 1966), insects (Salt, 1966) and nonhardy plant tissues (Kaku, 1964) can survive temperatures below -20° C if they remain subcooled but are killed at higher temperatures the moment ice forms.

These discoveries suggest that ice is the usual cause of death at subfreezing temperatures rather than loss of heat energy from cellular constituents. Most studies of freezing resistance of woody plants relate injury directly to temperature because formation of ice in tissues is difficult to measure. Low temperatures, however, irreversibly alter permeability (Ibanez, Casas and Redshaw, 1965) and reduce flexibility (Lyons, Wheaton and Pratt, 1964) of cellular membranes and disrupt metabolic processes (Christiansen, 1968) that cause injury to chilling sensitive plants between 0° and 7° C. Subfreezing temperatures as well as quantity of freezable water also determine rate of formation and quantity of ice in plant tissues. The vapor pressure gradient between extracellular and intracellular water increases with decreasing temperature (Mazur, 1969 and 1970) and energies of hydrophobic bonds of proteins and ordering of water

associated with proteins decrease with decreasing temperatures (Brandts, 1967). These factors favor loss of intracellular water and formation of extracellular ice but are offset by loss of permeability of cellular membranes with decreasing temperature (Moore, Davids and Berger, 1969). However, at slow cooling rates during equilibrium freezing of red blood cells (water flows from the protoplasm to maintain equilibrium between intra- and extracellular vapor pressure and prevents subcooling of intracellular water), rate of water loss doubles before falling to zero when little free water remains in the protoplasm.

Several investigators have recently followed the course of heat evolution and absorption during freezing and thawing of plant tissues with calorimetric measurements (Tumanov and Krasavtsev, 1959; Tumanov, Krasavtsev and Trunova, 1969 and Krasavtsev, 1969b) and freezing curve (McLeester, Weiser and Hall, 1968) measurements in order to express freezing resistance as a function of ice formation and to arrive at the source and cause of freezing injury. The experiment reported here was an attempt to relate seasonal change in freezing resistance to formation of ice in tissues of Douglas-fir.

Procedure

Terminal twigs from two Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) were collected at monthly intervals from September to May and frozen within five minutes in an ethylene glycol bath precooled to -18° C to provide a freezing rate of about 1° C per minute. In addition, twigs from bigcone Douglas-fir (Pseudotsuga

macrocarpa (Vasey) Mayr) and a *P. menziesii* x *P. macrocarpa* hybrid were sampled from December to March. A copper-constantan thermocouple of 30-gauge wire was inserted into the pith under the terminal bud of each twig and changes in temperature were recorded continuously on a Sargent dual channel potentiometer during freezing and thawing. Twigs were removed from the bath and thawed at room temperature (about 5° C per minute) after freezing to a predetermined position on the freezing curve in an attempt to relate injury to the quantity of ice and source of freezable water. Freezing curves were constructed by plotting tissue temperature during cooling as a function of time. Heat of crystallization of water at points of ice formation caused deflections in the curve that are characteristic of plant tissues. Injury to all major tissues was evaluated approximately two weeks after freezing by the technique described under Methods.

Temperature of the first subcooling point, first freezing point, second subcooling point, final temperature and time of freezing after incipient ice formation, and position of the freezing curve as a measure of ice formation upon removal of the cuttings from the freezing chamber were examined in a stepwise regression program to determine which variables were most closely related to injury. Injury was evaluated after cuttings were removed from the bath at the following positions of the freezing curve:

1. Before the first subcooling point or during incipient formation of ice if crystallization occurred during warming;
2. Immediately after incipient ice formation that raised temperature of the tissue to the first freezing point;

3. At the second subcooling point;
4. Midpoint of the plateau during formation of ice after the second subcooling point (at the second freezing point if present);
5. After rate of temperature reduction returned to normal and formation of ice was nearly complete.

Temperature at the first exotherm associated with freezing of the vegetative apex during January was assembled for each species. Results were evaluated by analysis of variance. Differences between means were determined by Duncan's new multiple range test (Harter, 1960). In addition, the statistical procedure was used to establish that temperature of the exotherm associated with death of the vegetative apex is a reliable index of freezing resistance.

Results and Discussion

Seasonal Variations in the Freezing Curve

The freezing curve for the stem of Douglas-fir under the terminal bud exhibited two subcooling points that are characteristic of plant tissues (Figures 49 through 53). It consisted of an initial subcooling point between -1° C and -9° C (usually between -3° C and -5° C), followed by a sharp exotherm to the first freezing point about 1° C higher; a rapid reduction in temperature to a second less distinct subcooling point at -6° C to -8° C; a long plateau at uniform temperature usually without a second freezing point; and a reduction in temperature at the normal cooling rate to small successive

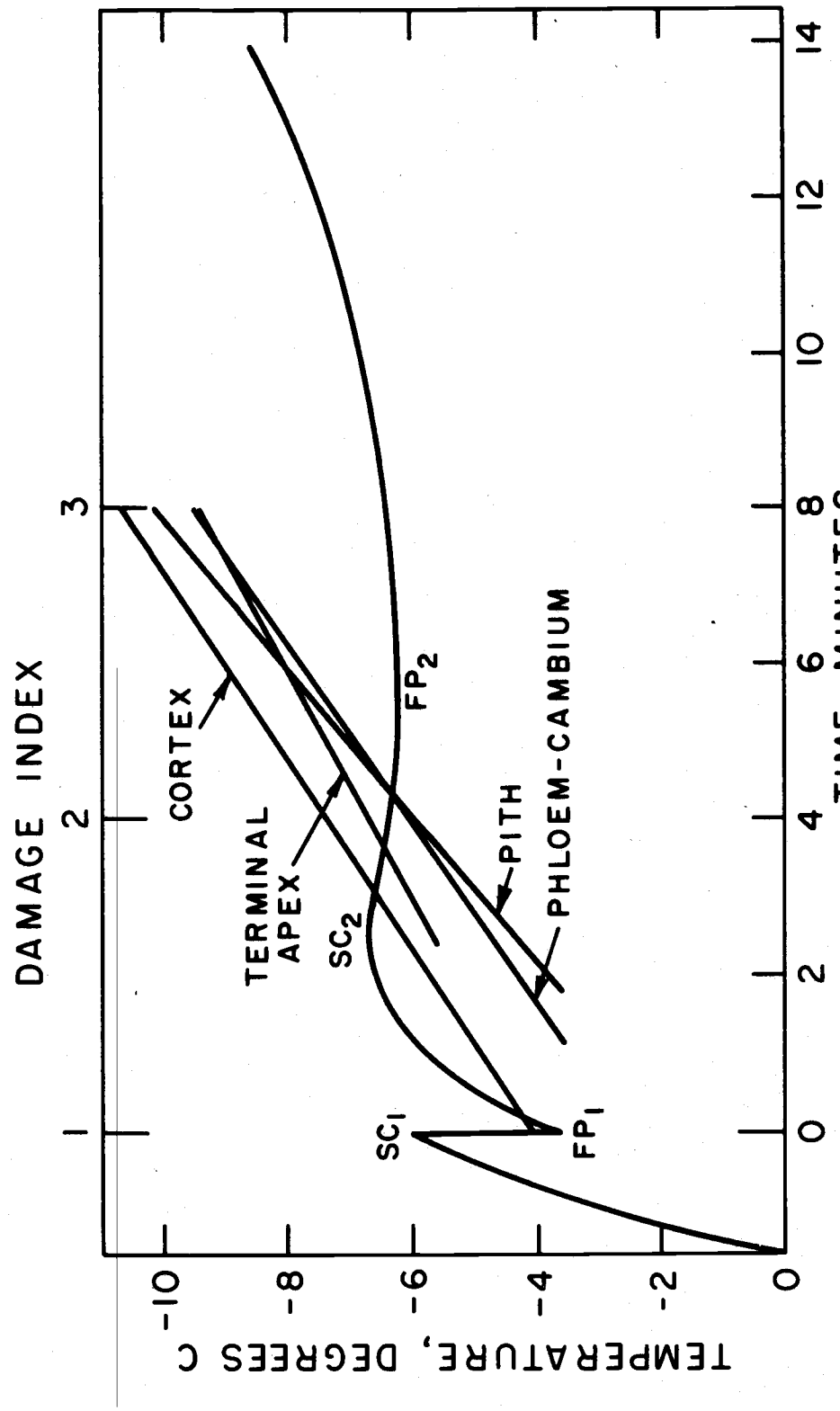

Figure 49. Average freezing curve of 11 unhardened terminal twigs from Douglas-fir on September 24. Injury was evident immediately after the first freezing point. Injury to each tissue is represented by the straight line regression between temperature and damage index.

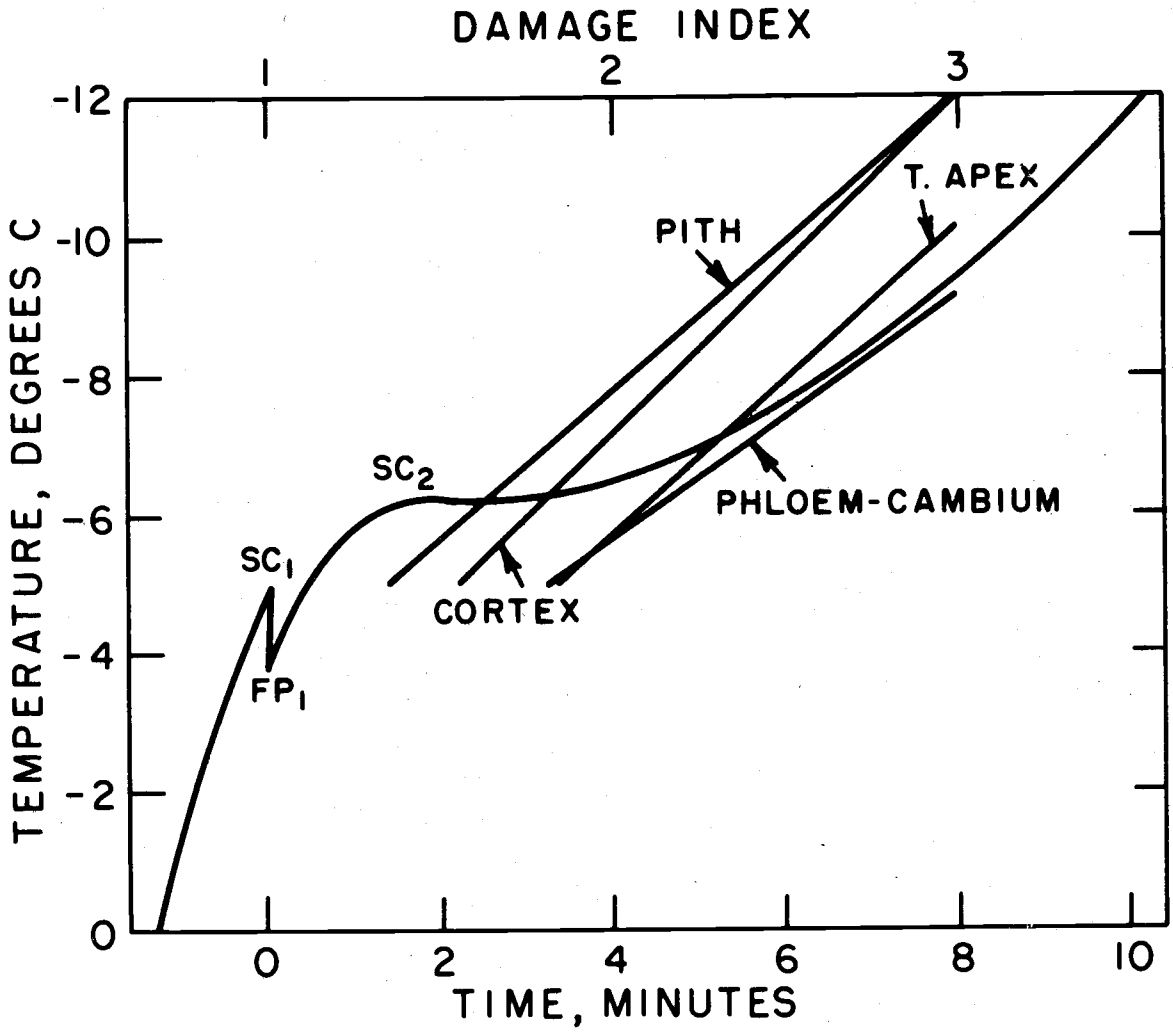

Figure 50. Average freezing curve of 23 terminal twigs from Douglas-fir on October 15. Tissues were in the early stages of hardening. Injury was evident after the first freezing point but before the second subcooling point. Injury to each tissue is represented by the straight line regression between temperature and damage index.

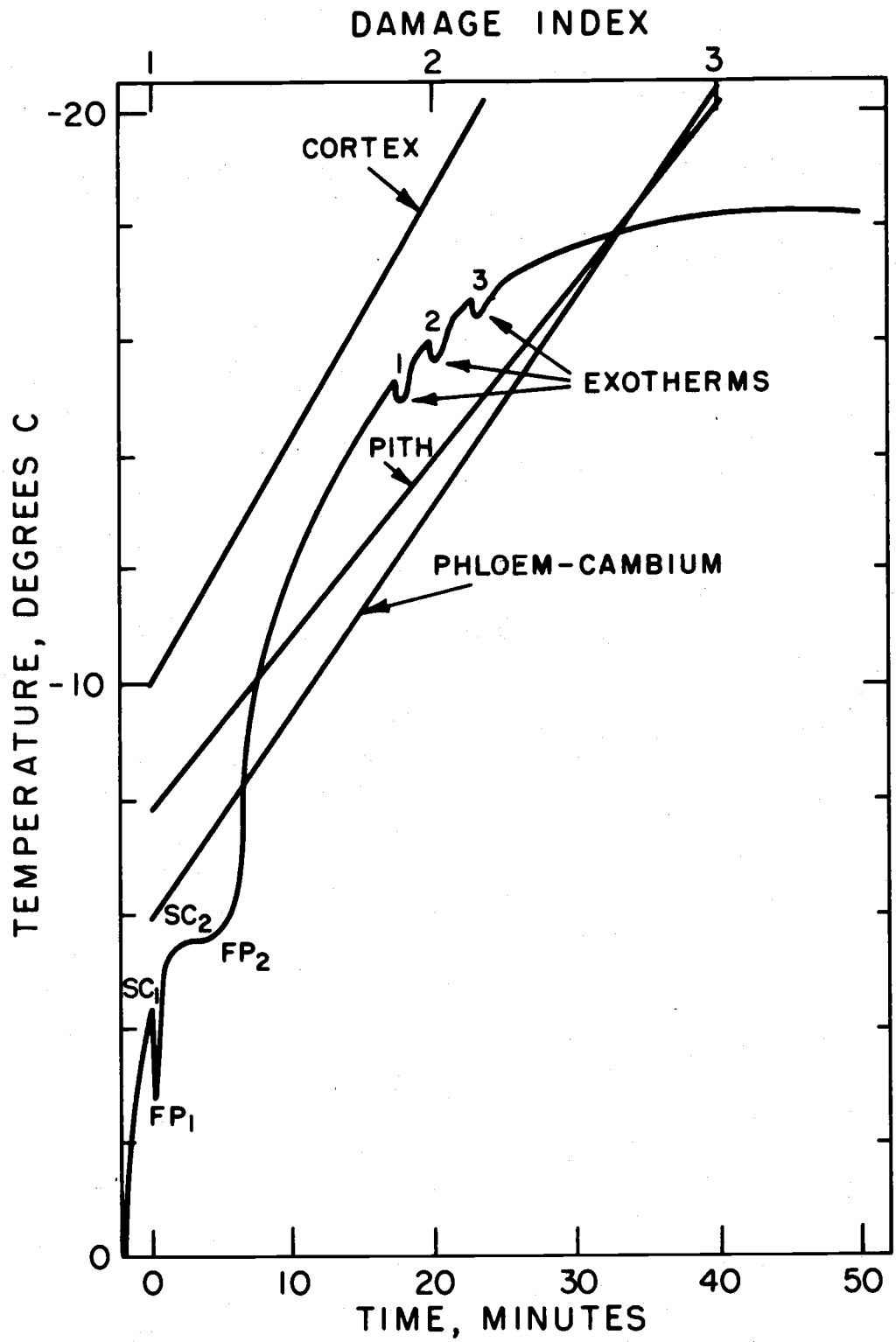

Figure 51. Average freezing curve of 17 hardened terminal twigs of Douglas-fir on December 12. Injury was evident after the plateau following the second subcooling point. Injury to each tissue of the stem is represented by the straight line regression between temperature and damage index. Exotherms 1 to 3 occurred with death of the terminal and lateral vegetative apices.

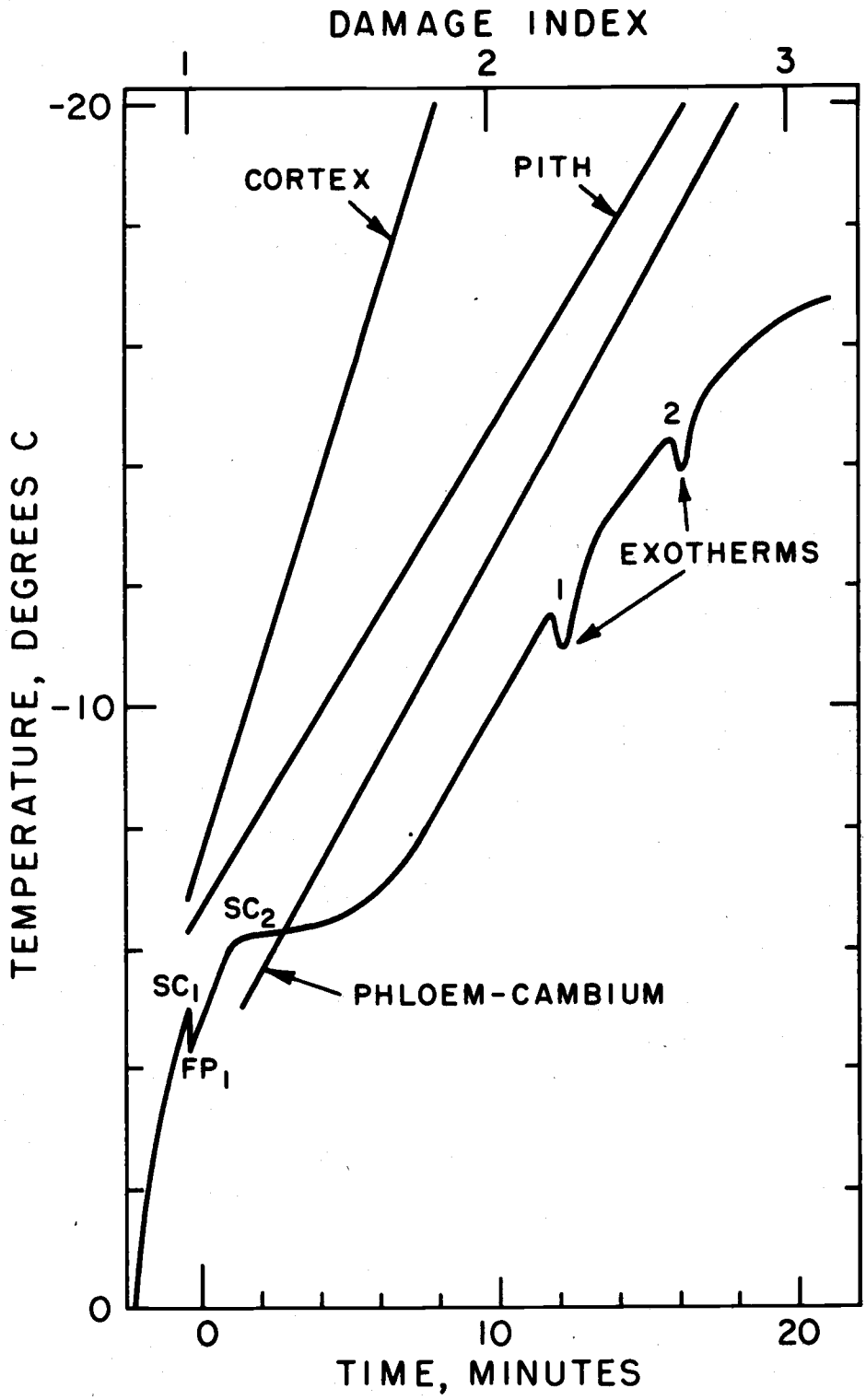

Figure 52. Average freezing curve of 24 terminal twigs of Douglas-fir on March 20. Tissues were rapidly dehardening. Injury was evident after the first freezing point. Injury to each tissue of the stem is represented by the straight line regression between temperature and damage index. Exotherms 1 and 2 occurred with death of the terminal and lateral vegetative apices.

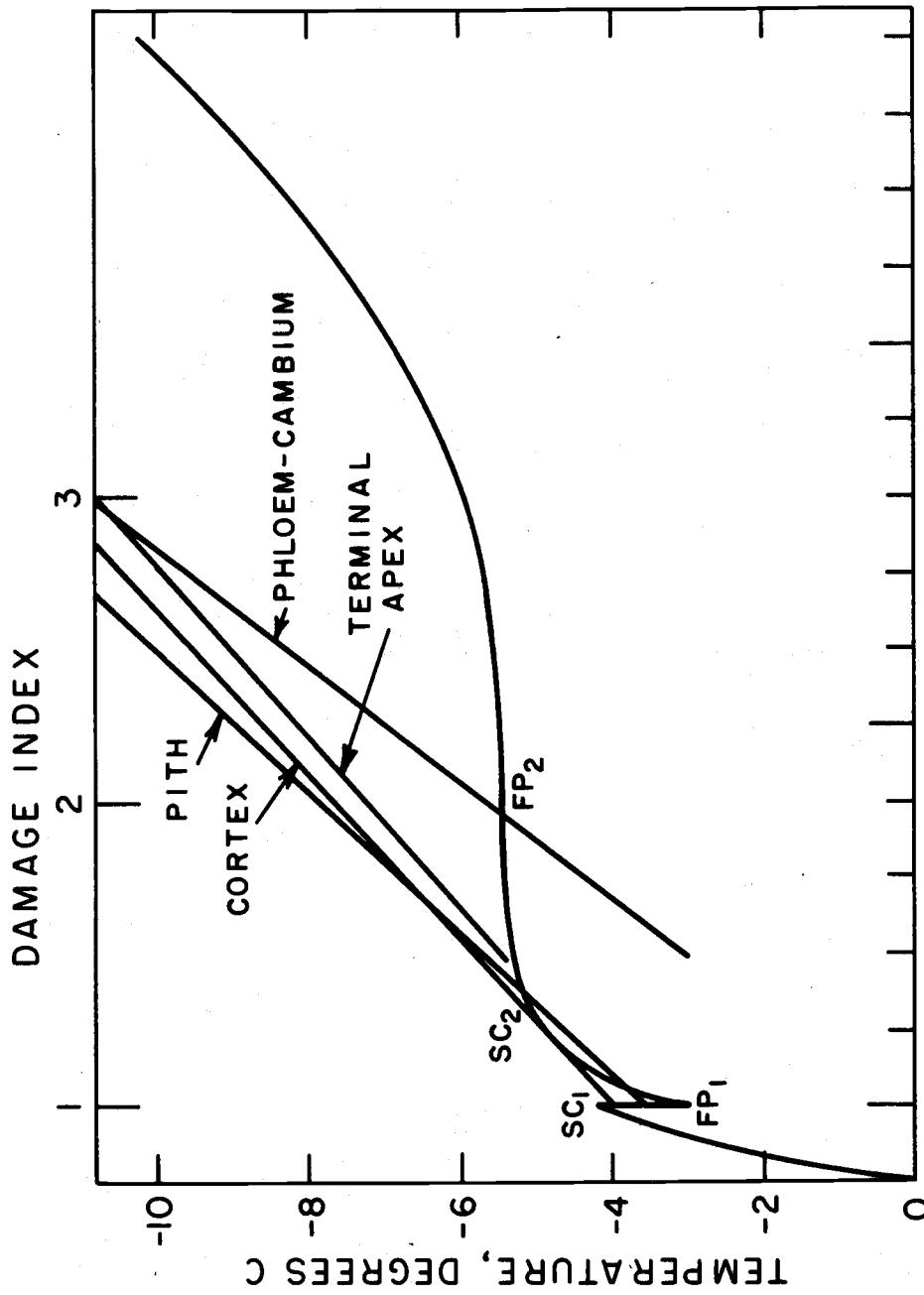

Figure 53. Average freezing curve of 11 terminal twigs from Douglas-fir on May 4 after loss of hardiness was nearly complete. Injury was evident after the first freezing point. Injury to each tissue is represented by the straight line regression between temperature and damage index.

exotherms that represented freezing of the terminal and lateral buds near the thermocouple. According to my definitions, subcooling points denote the lowest temperatures before rapid formation of ice while freezing points mark the highest temperatures after formation of ice. Exotherms are abrupt increases in temperature produced by heat of fusion of subcooled water. Both freezing points represent the highest temperature of tissues in the twig from heat of fusion of water and not the true freezing points of extra- and intracellular water.

The initial subcooling temperature for Douglas-fir was unpredictable and depended on such factors as internal and external moisture contents, cooling rate as determined by size of the twig and vibration in the freezing apparatus. External and internal moisture deficiencies decreased subcooling temperatures more than any other factor, however. Temperature of the first freezing point was positively correlated with both the first and second subcooling temperatures. Similar observations were reported by other workers (Modlibowska, 1962; Yelenosky and Horanic, 1969 and McLeester, Weiser and Hall, 1968 and 1969). Yelenosky and Horanic (1969) found that freezing points of citrus seedlings decreased less than initial subcooling points and difference between the two points increased with decreasing subcooling temperature. My interpretation of this observation is that the initial quantity of ice and heat of crystallization of water increased with decreasing subcooling temperature.

The second subcooling point in Douglas-fir was not always evident in my experiments at slow cooling rates (3^{o} C per hour) when

dissipation of heat of crystallization was slow. McLeester, Weiser and Hall (1969) found that rapid cooling rates (40° C per hour) gave better resolution of the second subcooling point for twigs with high water contents while slow cooling rates (5° C per hour) gave best resolution for dehydrated twigs.

I observed only one basic type of freezing curve for Douglas-fir. McLeester, Weiser and Hall (1968) found three basic freezing curves for twig sections of red-osier dogwood (Cornus stolonifera Michx.): A summer and winter curve with two freezing points; an early autumn curve with three freezing points, and a spring freezing curve with one freezing point that masked the second because of high moisture content and sap pressure. However, I observed a third exotherm when stem sections of Douglas-fir without needles and buds were frozen in December. A sharp but brief exotherm followed either the first or second subcooling point. This exotherm may have represented freezing of subcooled extracellular water of cells surrounding the thermocouple because it was abrupt and the freezing point increased 3° to 5° C.

Other variations in the freezing pattern of the twig of Douglas-fir included:

1. One to three small exotherms that were frequently evident just before the initial subcooling point during early autumn in particular. These exotherms are believed to represent freezing of extracellular water in tissues further than 0.5 cm away from the thermocouple;

2. Small size and susceptibility of the vegetative apex to injury may have prevented the appearance of exotherms associated with death of buds in early autumn before development of hardiness (Table XIII). Temperature of the exotherms gradually decreased during development of freezing resistance and increased during loss of hardiness in late winter. The exotherms enlarged and eventually merged with the plateau after subcooling point 2.

Table XIII. Seasonal change in mean temperature and time of subcooling of the vegetative apex after initial formation of ice in the stem cooled about 1° C per minute.

Date	Number of Exotherms	Mean Subcooling Temperature	Time After Initial Ice Formation(Min.)
September 24		Exotherms not evident	-
October 15		Exotherms not evident	-
November 12 - 13	16	-11.1° C	11.3
November 26 - 28	5	-14.2° C	20.2
December 10 - 11	11	-15.3° C	16.9
January 30	10	-17.4° C	21.3
March 20	8	-11.4° C	12.7
April 16	5	- 6.3° C	7.8 (exotherms not always evident)
May 4		Exotherms not evident	-

3. A reduction in length of the plateau following the second subcooling point during autumn indicated that freezable intracellular water declined during hardening. The plateau following the second subcooling point was greatly extended

after growth resumed in early spring because of high moisture contents and enlargement of the stem and vegetative apex. These observations suggested that the major decline in moisture during hardening and increase during loss of hardiness was in the inner space of tissues and not in freezable extracellular water.

McLeester, Weiser and Hall (1969) review theories proposed to explain double freezing points in plant tissues. Most researchers agree that the first exotherm represents crystallization of extracellular water and the second exotherm that produces the second freezing point or plateau after the second subcooling point represents freezing of water associated with the protoplasm. Salt and Kaku (1967), on the other hand, observed that the initial exotherm in needles of Colorado blue spruce (Picea pungens Engelm.) under moisture stress represented formation of ice in the xylem and the second exotherm represented freezing of the mesophyll. The time interval between the first and second exotherm was attributed to delayed propagation of ice through the endodermis to the mesophyll. However, McLeester, Weiser and Hall (1969) pointed out that homogenous tissues from storage organs have two subcooling points. They found that the delay between the first freezing point and second subcooling point of stems increased with increased moisture content, increased diameter of the stem and increased subcooling temperature before initial formation of ice (McLeester, Weiser and Hall, 1968 and 1969). Slow loss of heat after the first exotherm of twig sections larger than 5 mm in diameter increased the temperature of the first freezing point

and caused a plateau before reduction in temperature to the second subcooling point. Twigs smaller than 5 mm in diameter had no plateau between the first freezing and second subcooling points (McLeester, Weiser and Hall, 1969). Rapid and complete formation of ice after extensive subcooling of extracellular water reduced length of the plateau and also increased the temperature of the first freezing point.

The reason for delay in formation of ice between the first freezing point and second subcooling point in multiple tissues of Douglas-fir (Figures 49 through 53) and individual tissues is unknown. Perhaps dehydration of the plasma membrane and cell wall is necessary to increase permeability and diffusion of intracellular water to extracellular sites of ice formation. However, slow loss of heat from the stem or increased heat from high moisture content prolonged reduction in temperature to the second subcooling point. This indicated that the second subcooling point is temperature dependent and may depend on increased ratio of intracellular to extracellular vapor pressure at lower temperatures if water does not flow out of the cell. Destruction of cellular membranes and acceleration of intracellular formation of ice may have accounted for the increase in temperature to the second freezing point in September (Figure 49) before freezing resistance developed.

Injury in Relation to Ice Formation

Injury to tissues in all of my experiments usually depended more on lowest temperature after the first subcooling point than either progress of ice formation, or time of freezing from the first subcooling point to removal of the twig from the freezing chamber. Therefore, results were analyzed by a regression of injury against temperature (Figures 49 through 53). Each independent variable could be used to explain injury equally well, however, except when twigs were resistant and ice formation was nearly complete before injury was evident. Injury in relation to both progress of ice formation and time of freezing after intital formation of ice can be interpolated from the freezing curves (Figures 49 through 53).

Injury did not depend on temperature of the subcooling or freezing points and was not observed until after the first exotherm even in unhardened tissues. In addition, unhardened tissues frequently showed no evidence of injury if they were thawed within one minute after initial formation of ice but the pith, phloem-cambium and vegetative apex were often severely injured before ice formation was complete (Figures 49 and 50). This observation agreed with Yelenosky and Horanic's (1969) report that unhardened seedlings of citrus species survived occasionally if they were removed from the freezing chamber within one minute after initial formation of ice but the stems died back more than 90 percent if freezing continued for three to five minutes after the initial exotherm.

Freezing resistance of Douglas-fir increased from September

until December when injury occurred only after the second plateau of the freezing curve (Table XIV and Figure 51). Formation of ice may not have been complete even after the second plateau of the freezing curve although reduction in temperature of the stem returned to normal during freezing. Calorimetric measurements by Tumanov and Krasavtsev (1959) and Krasavtsev (1969) showed that freezing continued to -60° C in hardy plants.

The relation between injury and formation of ice was not consistent in early spring after loss of hardiness. Tissues of many twigs remained uninjured until formation of ice was nearly complete while others were injured immediately after the first exotherm. Ice may have formed in the xylem and dehydrated surrounding tissues of those twigs that remained uninjured. Recent studies of ice formation in plants (Hudson and Idle, 1962; Idle, 1966; Kitaura, 1967; Salt and Kaku, 1967; and Kaku and Salt, 1968) have shown that ice forms first in the xylem, then in surrounding tissues. Idle (1966) observed that ice may form in the cut surface of the cortex and central parenchyma before it forms in the vascular tissue of unhardened plants. If formation of ice in tissues becomes random during loss of hardiness and nucleation of ice is frequent in tissues susceptible to freezing injury, then dehydration and release of heat from freezing may prevent movement of ice into more resistant tissues.

Even unhardened stems of Douglas-fir were resistant to freezing of extracellular water because tissues frequently showed no evidence of injury if they were thawed rapidly after the initial

Table XIV. Seasonal change in freezing resistance of Douglas-fir in relation to progress of ice formation in the stem frozen at about 1° C per minute.

Date	Position of the Freezing Curve at Initial Injury [1/]			
	Cortex	Phloem-cambium	Pith	Terminal Apex
September 24	2	1	1	2
October 15	2	2	2	2
November 10	5	5	4	5
November 28	6	5	5	6
December 10	6	6	6	6
January 30	6	5	5	6
March 20	5	2	4	6
April 16	4	2	4	5
May 4	2	1	2	3

[1/] Position of the freezing curve:

1. Immediately after the first freezing point.
2. Between the first freezing point and second subcooling point.
3. At the second subcooling point.
4. At the second freezing point or during the plateau after the second subcooling point.
5. At the end of the second plateau.
6. After the second plateau.

exotherm. The ability of unhardened cells to survive freezing of extracellular water suggested that injury was caused by freezing of water associated with the protoplasm. However, many cells of unhardened tissues were injured before intracellular water was completely frozen (Figures 49 and 53). This observation established that complete dehydration of each tissue was not necessary for injury to unhardened cells.

In addition, injury that I observed before intracellular water was completely frozen suggested that cellular membranes or some other complement of the protoplasm that lost water early were chief sites of freezing injury in unhardened tissue of the stem if ice was confined to extracellular spaces. However, injury probably resulted from formation of intracellular ice. Intracellular ice must have formed in the vegetative apices that were killed from freezing because little or no extracellular space exists. Impermeability of immature membranes delays diffusion of water out of young cells and promotes supercooling and nucleation of intracellular ice (Levitt, 1956). High free water contents of unhardened tissues also favor intracellular freezing.

The decrease in length of the plateau after the second subcooling point during hardening indicated that free water in the protoplasm is reduced or incorporated into unfreezable structures that are associated with proteins or other macromolecules. This reduction in freezable water may be related to the autumn increase in macromolecular components of the protoplasm that is supposed to increase

the capacity of cells to withstand stresses from freezing (Siminovitch, 1963; Siminovitch et al., 1968 and Pomeroy, Siminovitch and Wightman, 1970). Brown, Bula and Low (1970) suggested that specific cytoplasmic proteins such as those of membranes may order water and reduce its partial molar free energy because hydration properties of water soluble proteins were not associated with hardiness of alfalfa.

Although unhardened tissues in the stem of Douglas-fir were injured in my experiments before freezing of intracellular water was complete, hardened tissues were injured only after most of the freezable water was removed from the protoplasm (Figure 51, Table XIII). This observation indicated that the site of injury from freezing may have been different in hardened and unhardened tissues. Development of freezing resistance must have included changes in the relation between water and freezing-sensitive components of the protoplasm that permitted dehydration without injury after freezing. Mazur (1969) pointed out that effects of dehydration by extracellular ice, more than any other factor, account for injury to tissues that avoid formation of intracellular ice. Physical alterations from dehydration that may irreversibly denature proteins include concentration of intracellular and extracellular solutes, changes in pH, reduced spatial separation of molecules and removal of structural water that stabilizes the native conformation and configuration of proteins.

Exotherms associated with death of the vegetative apices of Douglas-fir were not evident after loss of hardiness in early spring

in my experiments. However, new tissues of the apex remained uninjured until freezing in the stem progressed beyond the second subcooling point. This observation indicated that water associated with growing cells of the vegetative apex still subcooled to a lower temperature than water associated with cells of the stem. Immature cell walls such as those of the vegetative apex of Douglas-fir may lack nucleating sites for crystallization of water. Apparently nucleating agents are associated with cell walls of tracheids and vessels in hardy plants (Kaku and Salt, 1968; Kitaura, 1967; Hudson and Idle, 1962 and Idle, 1966).

The ability of the vegetative apex of Douglas-fir to subcool served as an index of freezing resistance for buds of hardy species. The subcooling temperature and time to freezing of the vegetative apex was consistently greater for Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) than for bigcone Douglas-fir (Pseudotsuga macrocarpa (Vasey) Mayr) and their putative hybrid frozen in January (Table XV). Karyotype of the putative hybrid has not been studied to verify the cross between P. menziesii and P. macrocarpa. However, freezing resistance of the vegetative apex was between the freezing resistance of the vegetative apices of P. menziesii and P. macrocarpa (Table XV). This indicates that the tree could be a hybrid. The difference in subcooling ability of the two species and their hybrid appeared to depend on the potential of the trees to begin growth in late winter. Bigcone Douglas-fir is highly susceptible to injury from late winter and early spring frosts (Dallimore and Jackson, 1948).

The subcooling temperature of reproductive apices of Douglas-fir may serve as an index of freezing resistance for ovulate conelets. Ovulate conelets enclosed by bud scales were also killed at the moment of ice formation before pollination in late winter. The subcooling temperature of fruit buds of peach trees has proved to be a satisfactory index of their freezing resistance (Johnston, 1922).

Table XV. Mean subcooling temperature and time from initial formation of ice in the stem to death of the first vegetative apex of two species of Douglas-fir and a putative hybrid frozen at 1° C per minute to -18° C on January 30. Five twigs were sampled for each species.

Species	Subcooling Temperature (Centigrade)	Time of First Exotherm After Initial Ice Formation (Minutes
P. menziesii	-17.7 A*	23.5 A*
P. macrocarpa	-14.3 B	13.6 B
P. menziesii x *P. macrocarpa*	-16.6 C	16.2 B

*No significant difference among means is indicated for those species sharing a common letter; statistical significance ($P < 0.01$) is indicated for species not sharing a common letter.

EFFECT OF TEMPERATURE, MOISTURE AND LIGHT ON LOSS OF HARDINESS AND REHARDENING IN LATE WINTER

The same environmental factors that promote freezing resistance and inhibit onset of growth of woody plants also delay loss of freezing resistance in early spring. Many conifers have a short period of winter dormancy and are very prone to loss of freezing resistance when temperatures increase in late winter (Khlebnikova, Girs and Kolovskii, 1963). During long exposures to low temperature, trees apparently undergo physiological changes that decrease their ability to reharden after freezing resistance is lost during thaws in late winter (Tumanov and Krasavtsev, 1955).

Some of my experiments showed that moisture content as well as temperature can influence the hardiness of Douglas-fir in late winter because excised twigs at 2° C without water increased in freezing resistance while twigs with water at 2° C decreased in freezing resistance (Tables X and XI). As a follow-up, I conducted several experiments to determine the effect of temperature, moisture and light on loss of freezing resistance and rehardening in March.

Procedure

Experiment 1. Twigs were collected on March 4 from six trees with severe potassium deficiency, six trees with moderate potassium deficiency, and six non-potassium deficient trees, and treated as follows to determine if low night temperatures prevent loss of hardiness and if Douglas-fir is capable of rehardening at 2° C after it loses hardiness in late winter.

Treatment 1. One cutting per tree was frozen at 3° C per hour to -18° C immediately after the collection was returned to the laboratory in order to establish the hardiness of each tree at the beginning of the experiment.

Treatment 2. One cutting per tree was placed in water at 25° C (room temperature) to deharden during the experiment.

Treatment 3. One cutting per tree was placed in water at 25° C for 12 hours during the day and at 2° C for 12 hours at night.

Treatment 4. One cutting per tree was placed in water at 25° C during the day for 11 hours but was allowed to dry one hour before it was transferred to a cold room at -9° C. All cuttings were enclosed in a styrofoam ice chest to delay heat loss and provide a slow rate of cooling to -9° C.

Treatment 5. One cutting per tree was placed in water at 25° C to deharden for four days and then transferred to continuous light in a cold room at 2° C.

Cuttings that received treatments 2 through 4 were cooled to -18° C at 3° C per hour after four days. Cuttings that received treatment 5 were rehardened at 2° C for two additional days before freezing to -18° C. Injury to tissues of the stem, foliage and buds of all twigs was evaluated ten days to two weeks after freezing.

Experiment 2. Cuttings from each of five trees collected on March 17 and maintained at 25° C (room temperature) were cooled at 3° C per hour to -14° C at 12 to 17 hour intervals after collection to determine the rate of dehardening. Cuttings maintained at 25° C

with and without water were frozen to -14° C to determine the effect of dehydration on loss of hardiness.

Experiment 3. Cuttings from two trees were dehardened for three-and-one-half days in water at 25° C on March 9. Samples of three cuttings per tree were subjected to the following treatments to determine the effect of hydration, light, subfreezing temperature and ice formation on rehardening.

Treatment 1. A sample of three cuttings from each tree cooled at 3° C per hour to -14° C immediately after returning to the laboratory on March 9 to determine the freezing resistance of each tree at the beginning of the experiment.

Treatment 2. A sample placed in water and continuous light at 20 foot-candles at 2° C immediately after returning to the laboratory on March 9. Freezing resistance of the sample was determined by cooling at 3° C per hour to -14° C after four days.

Treatment 3. A sample dehardened at 25° C for three-and-one-half days. Freezing resistance of the sample was determined by cooling at 3° C per hour to -14° C.

Treatment 4. A sample rehardened in darkness at -3° C for 49 hours. The severed end of each cutting was submersed in 1/8 inch of water before rehardening. During rehardening, ice in each vial nucleated tissues of the stem and prevented subcooling.

Treatment 5. A sample rehardened in darkness at -3° C for 49 hours without water in each vial to prevent nucleation of

ice in tissues of the stem.

Treatment 6. A sample rehardened in darkness at 2° C for two days with water.

Treatment 7. A sample rehardened in light at 2° C for two days with water.

Treatment 8. A sample rehardened in darkness at 2° C for two days without water.

Treatment 9. A sample rehardened in light at 2° C for two days without water.

Samples that received treatments 4 through 9 were also cooled at -3° C per hour to -14° C and warmed at 3° C per hour. Injury for all twigs was evaluated about ten days after freezing.

Results and Discussion

All tissues of twigs in water and exposed to 2° C at night in Experiment 1 lost hardiness after four days but not to the same extent as tissues of twigs maintained at 25° C (Table XVI). This result indicated that cool night temperatures delayed the loss of hardiness but did not prevent it when day temperatures were 25° C and tissues were fully saturated with water. The increase in hardiness of all tissues with stems removed from water before freezing to -9° C at night was not significant at the .95 probability level. Reduced moisture contents may maintain the high level of hardiness more than subfreezing temperatures at night because the same tissues failed to harden after four days at -9° C in late February (Tables X and XI).

Table XVI. Effect of low night temperatures on loss of hardiness and rehardening ability of Douglas-fir after four days in early March. Test temperature was -18° C and cooling and warming rates were 3° C per hour.

Treatment	Damage Index [1]					
	Needle		Stem			Vegetative Apex of the Terminal Bud
	Transfusion Tissue	Mesophyll	Cortex	Phloem-cambium	Pith	
1. Untreated	1.88	1.43	1.07	1.75^{ab}	1.54^{ab}	1.50^{ab}
2. Dehardened at 25° C	2.83^{b}	2.47^{b}	2.11^{b}	2.62	2.36	2.19^{c}
3. 25° C, day; 2° C night	2.49^{a}	2.11^{a}	1.54^{a}	2.25^{c}	1.96^{c}	2.33^{c}
4. 25° C, day; -9° C night	1.68	1.39	1.08	1.49^{a}	1.36^{a}	1.11^{a}
5. Dehardened, 4 days; rehardened at 2° C an additional 2 days	2.44^{a}	2.12^{a}	1.40^{a}	2.01^{bc}	1.78^{bc}	1.78^{bc}

[1] No significant difference among means is indicated for those treatments sharing a common letter; statistical significance ($P \leq 0.05$) is indicated for treatments not sharing a common letter.

Two days with continuous light and night temperature at 2° C restored some of the hardiness lost by tissues exposed to 25° C for four days. Only the terminal apex failed to reharden significantly at the .95 probability level after two days at 2° C. Analysis of variance indicated that tissues of the potassium-deficient and non-deficient trees responded equally to every treatment although all tissues of the nondeficient trees, except the phloem-cambium and terminal apex, were significantly (.95 probability level) less resistant that tissues of the deficient trees.

Results of the second experiment showed that hardiness of all tissues, except those of the stem within coriaceous bud scales of twigs without water, decreased during the first 17 hours at 25° C (Figures 54 through 56). Freezing resistance of all tissues, except the terminal and lateral vegetative apices of desiccated twigs, increased gradually, however, after the first 17 hours at 25° C. All tissues of twigs with water were killed or severely injured at -14° C after 17 hours at 25° C. These results indicated that moisture stress may have determined the freezing resistance of Douglas-fir in March when temperatures favor dehardening. A critical moisture level may have existed above which twigs dehardened rapidly at continuous high temperatures in the spring and below which injury decreased with increasing moisture stress after plants dehardened. However, van den Driessche (1969b) reported that moisture supplies between 70 and 100 percent of saturation had no effect on loss of hardiness of Douglas-fir seedlings potted in a peat and silica sand mixture. Li and Weiser (1969), on the other hand, found that removal

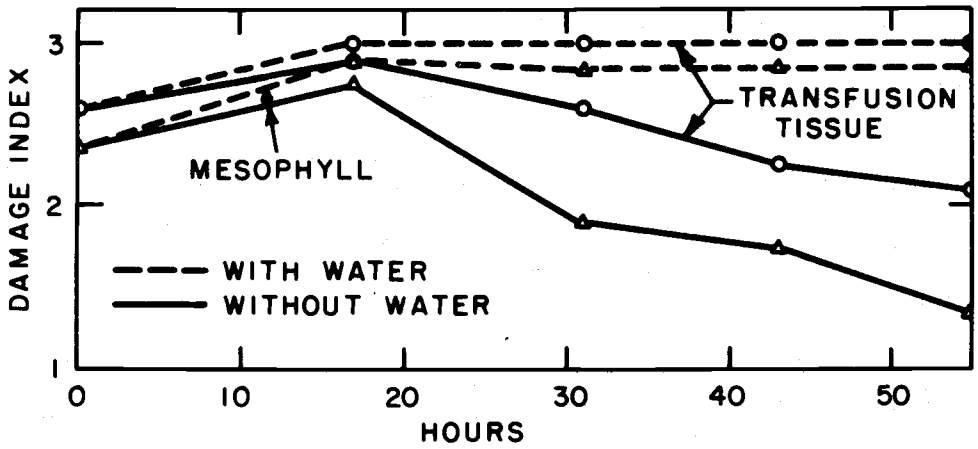

Figure 54.

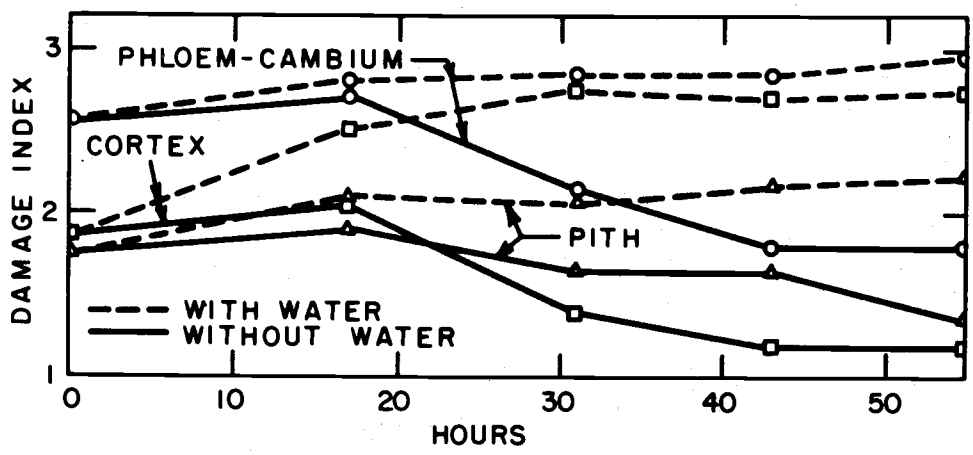

Figure 55.

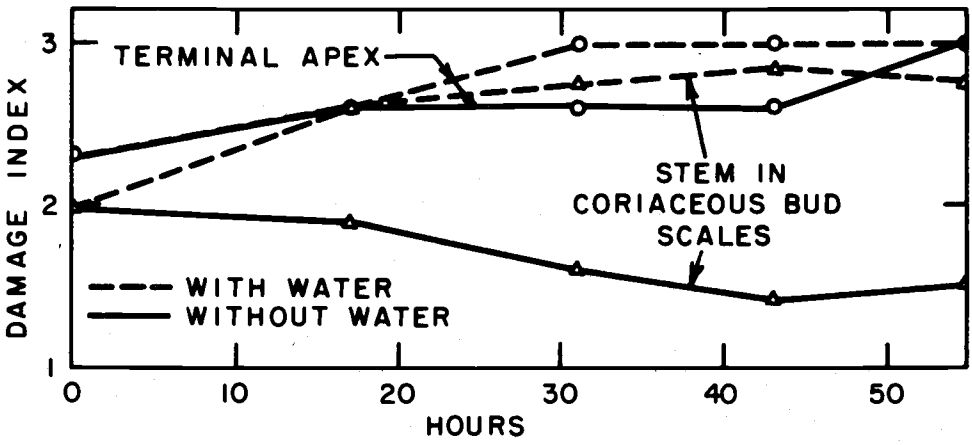

Figure 56

Figures 54 through 56. Effect of hours of dehydration at 25° C on injury to tissues of the foliage, stem and bud of Douglas-fir cooled to -14° C on March 17 - 19.

of only 4 to 14 percent of the water from actively growing stems of Cornus stolonifera Michx. produced a 2° to 6° C increase in hardiness, and removal of ten percent of the water produced a 12° C increase in freezing resistance of stems that were hardy to -21° C. Twigs from mulberry trees also increased in freezing resistance when desiccated for two or three days at 20° C in April (Sakai, 1960). Desiccation not only caused a decrease in water content but starch content decreased and sucrose content increased as well.

The cortex, tissues of the phloem-cambium and bud trace rehardened to a greater extent than other tissues of the twig when exposed to low temperature with several combinations of hydration and light (Table XVII). The decrease in frost resistance of all tissues of twigs in water at 25° C except the vegetative apices was significant after four days but tissues of twigs in water and light at 2° C for three-and-one-half days did not lose freezing resistance. One cutting sampled from twigs rehardened at -3° C without water to prevent nucleation of ice remained subcooled for fifty-and-one-half hours after 0° C and did not freeze until its temperature reached -7.7° C. A second cutting sampled from twigs rehardened at -3° C with water to induce nucleation of ice subcooled only two minutes after 0° C and ice formed at -1.7° C. Only the bud trace and transfusion tissue of twigs with ice, and the mesophyll, cortex, phloem-cambium and bud trace of twigs without ice, rehardened at -3° C. Vegetative apices of twigs at -3° C, on the other hand, lost freezing resistance without formation of ice in tissues of the stem. This result indicated that temperatures below 0° C caused injury to the vegetative apex

Table XVII. Effect of hydration, light, subfreezing temperature and formation of ice on rehardening of Douglas-fir on March 9 - 15. Test temperature was -14° C.

Treatment	Damage Index [1]							
	Needle		Stem			Buds		
	Transfusion Tissue	Mesophyll	Cortex	Phloem-cambium	Pith	Apices	Stem Within Coriaceous Scales of T. Bud	Trace
1. Untreated	2.17^{ab}	1.88^{ab}	1.00^{a}	1.92^{a}	1.54^{a}	1.17^{ab}	1.00^{a}	1.79
2. Hardened at 2° C	2.12^{a}	1.92^{ab}	1.21^{ab}	2.08^{ab}	1.88^{ab}	1.00^{a}	1.06^{a}	1.99
3. Dehardened at 25° C	2.75^{cd}	2.33^{b}	2.00^{d}	2.92^{de}	2.21^{b}	1.75^{ab}	1.83^{bc}	3.00^{c}
4. Rehardened at -3° C with ice	2.21^{ab}	1.75^{ab}	1.92^{cd}	2.62^{ce}	2.04^{ab}	1.50^{ab}	1.97^{c}	2.58^{ab}
5. Rehardened at -3° C without ice	2.25^{ac}	1.42^{a}	1.21^{ab}	2.33^{bc}	1.92^{ab}	2.67	1.40^{ac}	2.33^{a}
6. Rehardened at 2° C in darkness with water	2.54^{ad}	2.21^{b}	1.55^{bc}	2.75^{de}	2.33^{b}	1.37^{ab}	1.50^{ac}	2.54^{a}
7. Rehardened at 2° C in light with water	2.67^{bd}	2.25^{b}	2.17^{d}	3.00^{e}	2.29^{b}	1.87^{b}	1.89^{c}	2.92^{bc}
8. Rehardened at 2° C in darkness without water	2.88^{d}	1.83^{ab}	1.50^{bc}	2.58^{cd}	2.17^{b}	1.76^{ab}	1.22^{ab}	2.42^{a}
9. Rehardened at 2° C in light without water	2.42^{ad}	1.96^{ab}	1.17^{ab}	2.25^{ac}	1.96^{ab}	1.73^{ab}	1.34^{ac}	2.21^{a}

[1] No significant difference among means is indicated for those treatments sharing a common letter; statistical significance ($P < 0.05$) is indicated for treatments not sharing a common letter.

without dehydration by ice in the stem, and supported the earlier observation that subfreezing temperature and not formation of ice was the direct cause of partial injury to tissues of the apex after growth processes resumed in late winter. Failure of stems in water to reharden at -3° C may have resulted from prolonged presence of ice and high water content in tissues.

In contrast to the same treatment in the first experiment, all tissues of twigs in water failed to reharden at 2° C in continuous light and only the cortex and bud trace rehardened in darkness. Greater initial hardiness and less vigor of the potassium-deficient trees sampled in the first experiment may have delayed dehardening at 25° C and enabled tissues of twigs in water and continuous light to reharden at 2° C.

Only the pith, vegetative apices and tissues of the foliage of twigs without water failed to reharden at 2° C. Moisture content of two vegetative apices sampled from twigs in water at 2° C averaged 260 percent and three vegetative apices from twigs without water averaged 200 percent of dry weight before freezing to -14° C. Water present in the pith within coriaceous bud scales under the vegetative apices averaged 300 percent and 170 percent of dry weight at 70° C respectively. Analysis of variance of rehardening treatments at 2° C as a factorial experiment showed that light had no direct effect on rehardening of all tissues. A significant interaction between light and hydration status of the cortex and phloem-cambium tissues at the .99 and .95 probability levels suggested that light delayed rehardening of fully hydrated stems at 2° C and accelerated rehardening

of stems under moisture stress.

Cox and Levitt's (1969) hypothesis that growing plants fail to develop freezing resistance because low temperatures inhibit synthesis of hardiness promoting proteins uncoupled from growth processes at high temperature may explain the short term effect of light on rehardening of Douglas-fir twigs at 2° C. Light in conjunction with high moisture content at low temperature in my experiments with Douglas-fir may have prevented a shift from synthesis of proteins that promote growth to synthesis of proteins that impart hardiness. In addition, reduced pyridine nucleotides that prevent denaturation of proteins with sulfhydryl groups (Levitt, 1964 and 1967a) and are produced in the light reaction of photosynthesis may be oxidized at high moisture contents. Low moisture contents and darkness may prevent oxidation of pyridine nucleotides. Reduced triphosphopyridine nucleotide accumulates in hardened pea plants in light and remains at a high level in darkness for several hours at 25° C (Kuraishi et al., 1968). Reduced triphosphopyridine nucleotide is oxidized immediately in unhardened plants and fails to accumulate. Light also effects the internal water relation in some plants. Electrical impedance that reflected the water status of aqueous channels in cell walls of the stem and root of alfalfa increased after ten minute pulses of light at 4° C following ten hours of darkness (Hayden et al., 1969). Light decreased the impedance at 10° C, however.

DISCUSSION

The study of seasonal change in freezing resistance of tissues in the twig of Douglas-fir revealed a number of reasons why injury from freezing is difficult to evaluate. Freezing resistance of tissues varied during the year and differences in temperature between initial injury and death increased as tissues hardened. This pattern was similar to Kappen's (1968 and 1969) report that the difference in temperature between injury and death of the foliage of Douglas-fir was 2° C in summer and 6° C in winter. Tissues in my experiments had no resistance to injury from formation of ice before development of hardiness in late summer. Death of all tissues occurred immediately after subcooled extracellular water crystallized, usually at temperatures between -3° C and -6° C. In early winter, however, injury was not evident until nearly all water in the twig was frozen. Initial injury to the most susceptible tissues in hardened twigs such as transfusion tissue and interfascicular parenchyma of the bud trace began at about -15° C with slow cooling rates and all cells in these tissues were killed at -30° C. On the other hand, resistant tissues (cortex and bud scales) could be cooled to -30° C before injury became evident. For this reason, tolerance or survival of a plant cannot be predicted from freezing resistance of one organ or tissue. Lethal temperatures that kill 50 percent of the cells (LT 50) may be a poor index of survival because recovery of the plant often depends on resistance and regenerative ability of meristematic tissues.

In addition, the relative susceptibility of some tissues to freezing injury changed during development and loss of freezing resistance. The pith, for example, was more susceptible to injury than other tissues of the stem in autumn but more resistant in the spring. Such examples demonstrate that accurate estimates of survival and freezing resistance of plants or organs require knowledge of seasonal changes in relative tolerance of tissues and techniques to measure freezing injury of individual tissues.

The correlations between days to bud burst after March 31, potassium content of the current season's foliage, and freezing injury in the autumn and spring, are not proof of a causal effect of potassium nutrition and growth response on development and loss of freezing resistance. Nevertheless, tissues from trees that broke buds earliest in the spring had the highest potassium contents in the current season's foliage and were the first to develop freezing resistance in the autumn and lose freezing resistance in the spring. These observations suggested that potassium deficiency may impair the hardening mechanism of Douglas-fir in autumn and delay onset of growth and loss of freezing resistance in the spring. Studies with plants grown under controlled levels of potassium nutrition are needed to support this hypothesis.

Studies on the effects of low temperature, dehydration, and light on loss of hardiness and rehardening of tissues in late winter suggested that either environmental conditions unfavorable for growth or delayed onset of growth prevented loss of freezing resistance.

Ice in tissues of the bud with extracellular space dehydrated the vegetative apex and increased its ability to avoid formation of ice. Subcooling temperature of the vegetative apex declined from -16° C to -20° C as water content decreased from about 200 to 70 percent on a dry weight basis. Further reduction in freezable water may enable buds of Douglas-fir to survive temperatures of liquid nitrogen. The percentage of water retained by the apex during vitrification may provide an estimate of the amount and degree of orientation of quasi-crystalline water in the protoplasm and cell walls.

How water diffuses from the apex and remains subcooled in presence of ice in the stem of Douglas-fir was not clarified by my experiments. Cells of the crown may prevent seeding of ice from the stem into the vegetative apex as water moves to sites of extracellular ice by a mechanism of water movement in plant tissues. Freezing resistance of cells in the region of the lateral meristem could also depend on avoidance of ice. The lateral meristem may harden in response to loss of water to ice in the xylem. Analysis of x-ray diffraction patterns produced by frozen stem sections may identify sites of ice formation, free, and vitrified water.

The basic freezing curve for Douglas-fir frozen at 1° C per minute was characteristic of plant tissues in general. The freezing curve consisted of an initial exotherm that represented freezing of extracellular water and water in nonliving tracheids, a second prolonged exotherm that represented freezing of intracellular water, and a third exotherm that represented freezing of subcooled water in the vegetative apex. Except for the vegetative apices, injury of

tissues appeared to be a continuous process. Unhardened tissues survived freezing of extracellular water but were killed before freezing of intracellular water was complete. Injury based on the extent of browning in tissues appeared to increase as intracellular water was converted to ice. Hardened tissues, on the other hand, were injured only after most of the intracellular water was frozen. These observations indicated that:

1. Site of injury may be different in hardened and unhardened tissues.
2. The relations between water and freezing sensitive components of the protoplasm changed during hardening to permit severe dehydration without injury;
3. Injury to unhardened tissue was associated with freezing of intracellular water;
4. Complete dehydration of the protoplasm was not necessary for injury to unhardened tissues;
5. The chief sites of injury in unhardened tissues may be some complement of the protoplasm such as membranes that lose water early in the freezing process.

Some unhardened tissues may be subject to forms of injury other than dehydration at subfreezing temperatures. Ice appeared to rupture cell walls of the pith under the vegetative apex at slow rates of cooling in late summer and early autumn. This type of damage was mechanical. Partial injury to the rib and meristematic regions of vegetative apices after growth processes resumed in late winter may have been caused by low temperatures. Intracellular ice

did not cause partial injury of this type because previous trials had shown that formation of ice in the apex was complete in one minute or less after nucleation. Injury to the meristematic region and adjacent leaf primordia was occasionally evident in the unfrozen controls but to a lesser extent. The vegetative apices could have been injured in the field before the twigs were collected and either subfreezing temperatures or injury to vascular tissues from ice in the stem caused acceleration of breakdown processes.

Recent studies in controlled environments have shown that plants harden in response to low temperatures and short photoperiods. However, the first stage of hardening of Douglas-fir appeared to depend on formation and confinement of ice in tissues that are not easily injured by freezing. Thus early stages of hardening in new twigs may depend on differentiation of tissues and development of empty tracheids and intercellular space for ice as well as biochemical changes that enable the protoplasm to survive stresses from freezing.

Injury to hardened tissues of Douglas-fir at both slow and rapid cooling rates gradually increased with decreasing temperature and time of exposure after ice formation was nearly complete. This observation did not agree with Weiser's (1970) report that stem tissues are killed at the moment of freezing. Weiser observed a third exotherm similar to that for the vegetative apex of Douglas-fir and simultaneous death of the stem. I am more inclined to believe that the third exotherm may result from freezing of subcooled water in cells of the cambium region that do not have extracellular space. Injury to individual cells may be discontinuous, but in my experiments

variation in browning two weeks after freezing suggested that injury of hardened tissue with extracellular space is continuous over a wide temperature range. For example, initial injury to the cortex of hardened twigs frozen at 1° C per minute was observed at -12° C while partial injury occurred at temperatures lower than -20° C.

How plants acquire freezing resistance and the manner in which subfreezing temperatures cause injury to plant tissue have remained largely unresolved. The results of this thesis support the hypothesis that injury to hardened tissues is caused by ice and not low temperatures. Further investigations of effects of low temperature and rate of temperature change on structure of water and ice in plant tissues may help to decide the validity of this hypothesis. Knowledge of water structure in plants, function of water in maintaining the integrity of structural molecules of protein nature, effects of dehydration by extracellular ice on these molecules, and the mechanisms of water movement between or within tissues are probably important aspects of the cold resistant mechanism in plants. Techniques developed to measure water potential of plant tissues have not been used in studies of freezing resistance. These techniques in conjunction with freezing curve and calorimetric studies may provide information about water retaining forces of tissues during hardening and freezing, and ultimately contribute to a better understanding than we have of the complex processes involved in acquisition and loss of cold hardiness.

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APPENDIX

Appendix Table I. Percent potassium content of the current season's foliage and date of flushing for potassium-deficient Douglas-fir.

Tree[2]	January K Content Percent	March K Content Percent	Date of Flushing[1] South Aspect	North Aspect
1	.12	.16	26	32
2	.24	.26	32	39
3		.33	21	32
4		.37	13	18
5		.33	40	46
6	.13	.13	26	36
7	.15	.17	32	40
8	.15	.16	31	40
9	.13	.12	24	38
10	.12	.20	26	32
11	.16	.22	13	33
12	.13	.17	27	40
$\bar{X}$ and .95 conf. intvl.	.15 ± .03	.22 ± .05		
A_s	.13	.22	32	39
B_s	.16	.25	30	40
C_s	.22	.23	31	40
D_s	.13	.24	32	40
E_s	.13	.17	32	41
F_s	.15	.20	33	45
G_s	.16	.27	20	34
H_s	.13	.21	29	38
I_s	.16	.25	25	37
$\bar{X}$ and .95 conf. intvl.	.15 ± .03	.23 ± .02		
A	.25	.32	13	32
B	.30	.49	27	32
C	.34	.45	11	32
D	.24	.23	29	36
E	.27	.31	27	32
F	.17	.25	32	41
G	.20	.30	23	38
H	.25	.35	20	39
I	.30	.42	24	29
$\bar{X}$ and .95 conf. intvl.	.26 ± .04	.35 ± .07		

[1] Days after March 31.

[2] Letters with the subscripts designate trees with symptoms of potassium deficiency.

Appendix Table II. Percent potassium content of the current season's foliage and date of flushing for potassium-nondeficient Douglas-fir.

Tree	January K Content Percent	Days to Flushing After March 31 South Aspect	North Aspect
2	.52	19	19
3	.37	26	29
4	.41	15	24
5	.49	20	29
6	.61	37	41
7	.50	17	18
8	.47	20	24
9	.49	29	35
10	.49	22	27
11	.48	13	16
12	.68	26	33
13	.43	28	34
14	.46	28	31
15	.71	24	29
16	.61	36	37
17	.50	10	13
18	.46	28	30
$\bar{X}$ and .95 conf. intvl.	$.51 \pm .05$		