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FROST HARDINESS OF CONIFEROUS SEEDLINGS: PRINCIPLES AND APPLICATIONS

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ABSTRACT--The external factors that influence frost hardiness such as temperature, light, moisture and nutrients are examined as well as the internal biochemical changes that are associated with hardiness. Several types of freezing such as intra- and intercellular freezing are identified and its resulting injury. It is shown that frost hardiness occurs in two stages and that differential frost hardiness between various types of tissue is important, particularly when assessing injury.

The various methods for testing frost hardiness are examined. Those that have practical application, and are used operationally such as the detailed browning test (whole seedling assessment) and electrical conductivity method are discussed in detail as are the semi-operational electrical impedance methods. Methods that have operational potential, but require further development are identified, with particular emphasis on the differential thermal analysis (DTA) technique.

The importance to the tree grower of frost hardiness and the practical applications of this basic knowledge is discussed in sufficient detail so that it can serve as a guideline for frost hardiness testing.

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9.1 PRINCIPLES

9.1.1 Frost Hardiness Defined

There are several definitions of frost hardiness. It can be defined as the lowest temperature below the freezing point to which a seedling can be exposed without being damaged. It is also frequently defined as the minimum temperature at which 50 percent of the seedlings are killed, which is expressed as lethal temperature 50 (LT50).

The purpose of the frost hardiness testing will determine which definition is most appropriate. When testing to find the lowest temperature at which no damage occurs then the first definition is most suitable. But when testing to determine the temperature at which 10 or 50 percent damage occurs (i.e. "LT10" or "LT50") then the second definition is most appropriate.

The phenomenon which enables a tree to increase or decrease its resistance to cold is called the frost hardiness process. In the literature this process is also referred to as cold acclimation, acclimation, winter or cold hardiness or cold resistance.

9.1.2 Importance of Frost Hardiness

The frost hardiness process provides trees with the ability to survive the winter in temperate and cola climates. Thus, trees native to these climates possess the ability to harden and deharden, while many of the trees in sub-tropical and tropical climates do not.

In temperate and cold climates the natural frost hardiness process is expressed by the hardening and dehardening, which occurs in concert with the seasons (Figs. 1 and 2). This poses few problems, except for some late spring and early fall frosts that result in damage of varying degrees of severity but rarely total damage. However, in our efforts to grow trees in nurseries, both out-of-doors and in greenhouses, an understanding of the frost hardiness process is a necessity. It will assist us in manipulating nursery and greenhouse environments so that we can produce properly hardened stock.

With the great variety of stock types that are produced in nurseries and the diversity of nursery cultural practices associated with these stock types the importance of frost hardiness cannot be overemphasized. It is needed when you: 1/ use overwinter frozen storage, 2/ produce large numbers of containerized seedlings for overwintering outside and which have been grown (forced) under heavy fertilization schedules, 3/ introduce exotic tree species, and 4/ hybridize both native and exotic species.

Actively growing trees are not frost hardy, and before these trees can become totally hardy, growth has to stop and dormancy has to set in. Bare-root stock should not be lifted in the fall for overwinter frozen storage until both the shoot and root of the stock are sufficiently hardy. This is just as important when over-wintering container stock outdoors, whose root systems will be above the ground and surrounded by the cold circulating air, rather than the relatively warm insulated environment of the soil. It means that we must use the right nutrient regime (or any other cultural practice) to ensure that all growth has stopped before inclement conditions prevail.

When growing hybrids and exotics some answers are required to some pertinent questions, such as, a/ is their maximum winter hardiness high enough for the location? b/ do they become frost hardy early enough in the fall so that they can keep ahead of the decreasing temperatures?, and c/ do they retain their frost hardiness in the spring long enough so that a prolonged mild spell in late winter or early spring does not result in early dehardening, which can damage or kill them?

9.1.3 Mechanism of Freezing and Freezing Injury

Two types of freezing are recognized to occur in plants and they are characterized by the location of ice formation in the plant tissues. When water freezes inside the cell, it is called intracellular freezing, but when it freezes outside the cells, in the intercellular spaces, it is referred to as intercellular or extracellular freezing. Intracellular ice formation is with few exceptions considered to be lethal (Mazur 1977, Lyon *et* al. 1979). It is lethal regardless of the hardiness of the tissue or plant and is caused by rapid decreases in temperature, such as greater than 10 Celsius degrees per minute. Intracellular freezing seldom occurs under natural conditions and therefore rarely concerns us. On the other hand, moderate decreases in temperature (i.e. 1 to 6 Celsius degrees per hour) cause intercellular ice formation, which may or may not be lethal, depending on the hardiness of the plant. Intercellular ice formation, therefore, concerns us greatly.

When temperatures go below the freezing point (0'C), the water between the cells will freeze first, forming ice crystals. Under natural conditions, some supercooling will occur down to as low as -7'C. Following the initial ice crystal formation, water will be drawn out of the cells to the enlarging ice crystals, causing the cells to shrink while the water inside

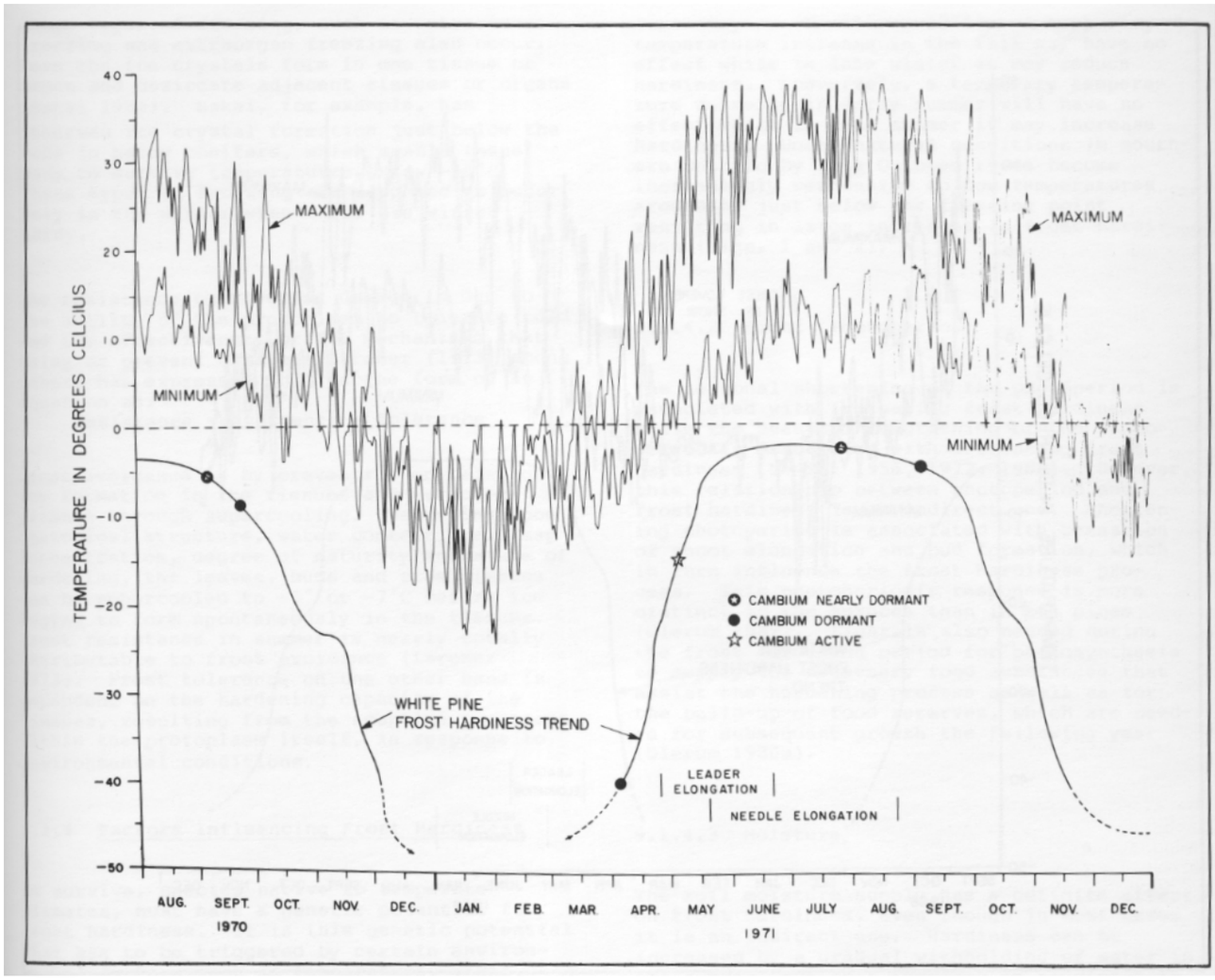

FIGURE 1 A WHITE PINE (PINUS STRUBUS L.) SEASONAL FROST HARDINESS TREND AND THE DAILY MAXIMUM AND MINIMUM AIR TEMPERATURES MEASURED AMONG THE TREES DURING THE COURSE OF THE EXPERIMENT. THE FROST HARDINESS TEMPERATURE DELINEATED IS THE LOWEST TEMPERATURE TO WHICH TREES CAN BE EXPOSED WITHOUT BEING DAMAGED. THE FROST HARDINESS INCREASED FROM LATE AUGUST UNTIL ITS MAXIMUM HARDINESS, WHICH IS BELOW -40°C IN DECEMBER. DEHARUENING STARTED IN MARCH AND WAS RAPID DURING APRIL AND PART OF MAY. THE MINIMUM FROST HARDINESS OF ABOUT -3°C WAS REACHED BY THE END OF MAY. THE FROST HARDINESS STARTED TO INCREASE AGAIN TOWARDS THE END OF JULY WHEN THE CAMBIUM STARTED TO BECOME DORMANT AND INCREASED SLOWLY AT FIRST AND THEN RAPIDLY TOWARDS ITS WINTER MAXIMUM. THE RATE OF HARDENING LEVELLED OFF BETWEEN -20°C AND -25°C IN BOTH OCTOBER 1970 AND 1971 BUT WAS MORE PRONOUNCED IN 1970 THAN IN 1971. THE TREND IS TRUNCATED AT -40°C (DECEMBER TO MARCH) BECAUSE IT WAS NOT POSSIBLE TO CONDUCT FREEZING TESTS BELOW -40°C. VISIBLE SIGNS OF GROWTH OCCURRED WELL BEFORE THE TREES HAD TOTALLY DEHARDENED. THE 3- TO 4-YEAR-OLD TREES WERE GROWN IN POTS AND TESTED INTACT NEAR MAPLE, ONTARIO (LAT. 43°51'N, LONG. 79°31'W).

the cell remains unfrozen. It is this dehydration of the cell that is now considered the fundamental cause of freezing injury.

During this cellular dehydration there is a simultaneous 1/ decrease in cell volume and surface area, reducing the volume to surface area ratio (i.e. the cell shrinks), 2/ an increased concentration of cell solutes, 3/ pH changes of the cellular sap caused by the precipitation of some buffering salts and 4/ the removal of hydration water from macromolecules (Lyon et al. 1979). For many years

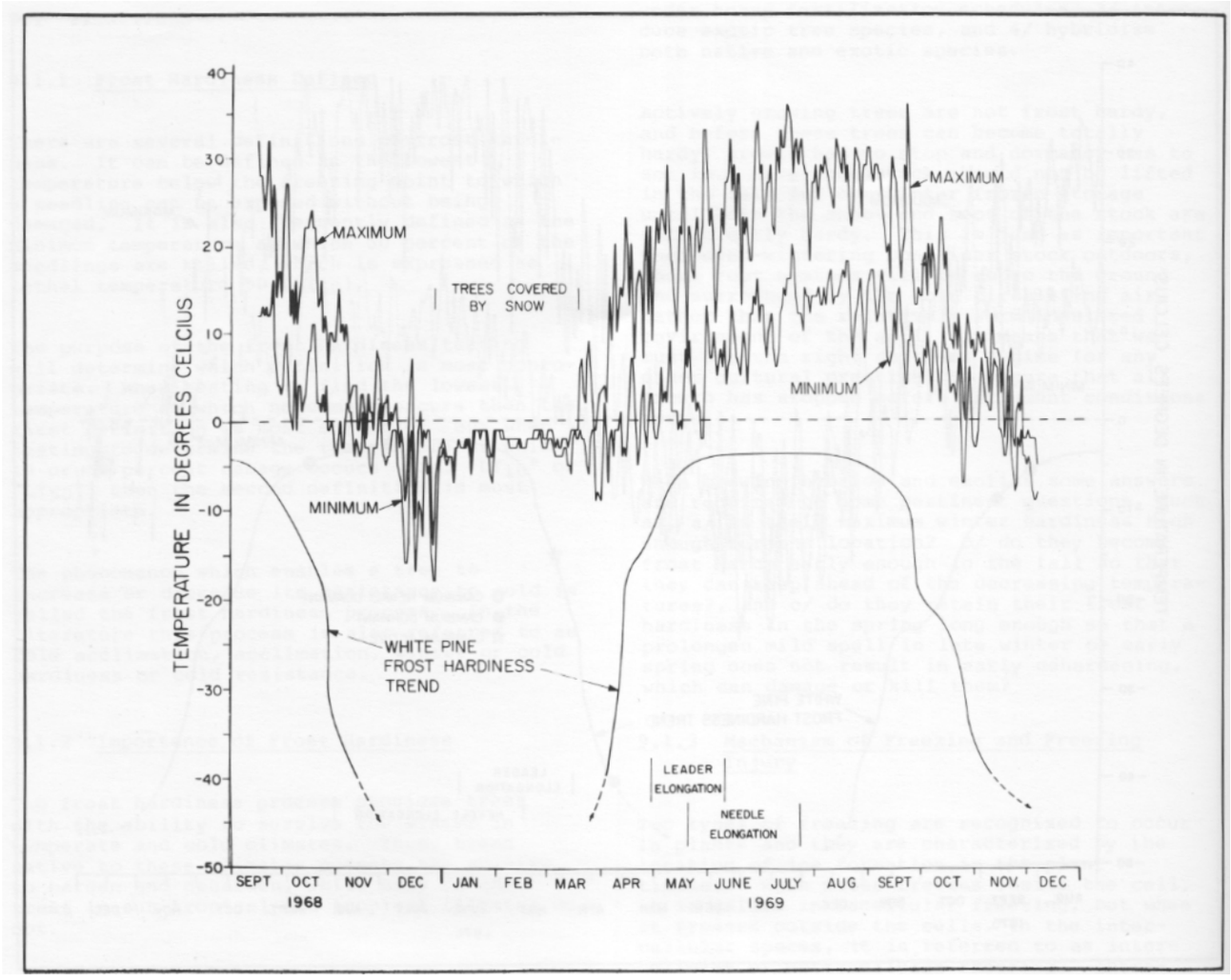

FIGURE 2 A WHITE PINE SEASONAL FROST HARDINESS TREND AND THE DAILY MAXIMUM AND MINIMUM AIR TEMPERATURES MEASURED AMONG THE TREES DURING THE COURSE OF THE EXPERIMENT. THE FROST HARDINESS INCREASED FROM LATE AUGUST UNTIL ITS MAXIMUM HARDINESS, WHICH IS BELOW -40'C, AT THE END OF NOVEMBER. DEHARDENING STARTED IN APRIL AND WAS RAPID FOR THE FIRST TWO WEEKS AFTER WHICH IT BECAME MORE GRADUAL. THE MINIMUM FROST HARDINESS OF ABOUT -3'C WAS REACHED BY THE END OF MAY. THE FROST HARDINESS STARTED TO INCREASE AGAIN IN AUGUST AND REACHED ITS WINTER MAXIMUM IN NOVEMBER. THE RATE OF HARDENING LEVELLED OFF AT BETWEEN -20'C AND 25'C FOR APPROXIMATELY A WEEK IN OCTOBER 1969. THE TREND IS TRUNCATED AT -40'C (NOVEMBER TO MARCH) BECAUSE IT WAS NOT POSSIBLE TO CONDUCT FREEZING TESTS BELOW -40'C. THIS HARDENING TREND HAS BEEN DISCUSSED, UNILLUSTRATED, PREVIOUSLY (GLERUM 1973b).

most frost hardiness scientists thought that these solution effects of dehydration were the cause of the freezing injury (Steponkus 1978). However, it is now generally accepted that the primary effects of freezing are due to membrane disruption (Lyon et al. 1979, Steponkus 1984). Ice formation occurs in both hardy and nonhardy tissues, but the hardy tissues survive, while the nonhardy tissues do not. The hardy plant is able to protect all the cell membranes from the effects of freezing, which is accomplished through a combination of chemical protection and membrane synthesis.

Other types of freezing, such as extratissue freezing and extraorgan freezing also occur. Here the ice crystals form in one tissue or organ and desiccate adjacent tissues or organs (Sakai 1982). Sakai, for example, has observed ice crystal formation just below the buds in hardy conifers, which enable these buds to survive temperatures below -50'C. These types of freezing are supposed to occur only in the winter when trees are winter hardy.

The resistance to freezing damage is due to the ability of the protoplasm to tolerate cold and the effectiveness of the mechanisms that delay or prevent damage. Larcher (1973) among others has expressed this in the form of an equation as:

Resistance - Avoidance + Tolerance

Frost avoidance is by prevention or delay of ice formation in the tissues and is accomplished through supercooling. Depending upon anatomical structure, water content, cell-sap concentration, degree of maturity and state of hardening, the leaves, buds and stem tissues can be supercooled to -5° or -7'C before ice begins to form spontaneously in the tissues. Frost resistance in summer is nearly totally attributable to frost avoidance Marcher 1973). Frost tolerance on the other hand is dependent on the hardening capacity of the tissues, resulting from the adaptive processes within the protoplasm itself, in response to environmental conditions.

9.1.4 Factors Influencing Frost Hardiness

To survive, species native to temperate climates, must have a genetic potential for frost hardiness. It is this genetic potential that has to be triggered by certain environmental factors such as temperature, light, moisture and nutrients, before it can be expressed. The interactive effects of these factors make it difficult to evaluate the effects of a single factor (Van Den Driessche 1969). However, since all these factors can be manipulated to varying degrees by the nurseryman depending on whether the stock is grown out-of-doors or in greenhouses, they should be examined separately. It is now generally agreed by frost hardiness investigators that the main environmental factors that trigger the frost hardiness process are temperature and light (photoperiod).

9.1.4.1 Temperature

Decreases in seasonal temperatures are accompanied by frost hardiness increases and seasonal temperature increases are accompanied by frost hardiness decreases (Figs. 1 and 2). Temporary changes in temperature have different effects on frost hardiness depending on time of year (Vasil'yev 1956). A temporary temperature increase in the fall may have no effect while in late winter it may reduce hardiness. Conversely, a temporary temperature decrease in early summer will have no effect while in late summer it may increase hardiness. Under natural conditions in southern Ontario by late October trees become increasingly responsive to low temperatures around or just below the freezing point resulting in large increases in frost hardiness (Figs. 1 and 2).

9.1.4.2 Light (Photoperiod)

The seasonal shortening of the photoperiod is associated with increasing frost hardiness while the seasonal lengthening of the photoperiod is associated with decreasing frost hardiness (Levitt 1956, 1972, 1980). However, this relationship between photoperiod and frost hardiness is an indirect one. Shortening photoperiod is associated with cessation of shoot elongation and bud formation, which in turn influence the frost hardiness process. This photoperiodic response is more distinct in the spruces than in the pines (Glerum 1982). Light is also needed during the frost hardening period for photosynthesis to supply the necessary food substances that assist the hardening process as well as for the build-up of food reserves, which are needed for subsequent growth the following year (Glerum 1980a).

9.1.4.3 Moisture

The soil moisture supply has a definite effect on frost hardiness, even though in most cases it is an indirect one. Hardiness can be increased by a gradual withholding of water in the summer, but this increase in hardiness is more the result of growth cessation than just the reduction in water supply. A prolonged lack of water (i.e. drought) in the summer actually has an adverse effect on hardiness (Fraser and Farrar 1957). In essence, stresses of any kind, including moisture stress, should be avoided as they will interfere with physiological processes, that in turn interfere with the frost hardiness process. The standard horticultural practice of watering conifers adequately in the fall so that they winter well, clearly implies that moisture stress should be avoided during the hardening period.

9.1.4.4 Nutrients

Adequate nutrient supplies are needed if the frost hardiness process is to be effective. Plants always winter better on fertile soils, which are properly cultivated and fertilized than on soils which are poorly cultivated and

unfertilized (Vasil'yev 1956). The affects of nutrients on frost hardiness are ambiguous. In general nitrogen is supposed to reduce hardiness or prevent the plant from becoming frost hardy while potassium and phosphorus are supposed to increase hardiness (Levitt 1956). Although there are many observations to support this generalization there are also many observations which do not support it (Aronsson 1980; Christersson 1973, 1975; Levitt 1980). The many contradictory results in nutritional studies are due in large part to the fact that nutrients stimulate or reduce the rate of growth and thus affect frost hardiness indirectly. Nitrogen stimulates growth and when applied late in the growing season can prevent growth cessation, bud development and subsequent frost hardening. However, it can also improve hardening when applied after budset (Colombo and Glerum 1983, unpubl. data). Some other observations tend to support our findings (Anderson and Gessel 1966, Benzian et al. 1974). Potassium and phosphorus on the other hand can assist in growth cessation and bud development. What are important are the amounts of nutrients, their relative proportion to each other and the timing of their application.

These environmental factors influence dormancy as well as frost hardiness and are an important component of my premise that in many tree species native to temperature and cold climates a relationship exists between dormancy and frost hardiness (Glerum 1973a, 1976, 1982). This premise is supported by other obervations on dormancy and frost hardiness (Larcher 1982). In my view, this relationship is of great importance to forest nursery practice but its discussion is beyond the scope of this paper.

Environmental factors affect physiological processes only by changing internal conditions and processes. In the field of plant physiology this is generally known as Kleb's concept (Kramer and Kozlowski 1979) and the frost hardiness process is no exception.

9.1.5 Internal Changes Associated with Frost Hardiness

When the frost hardiness process is activated and the tree goes from its summer condition of minimum frost hardiness to its winter condition of maximum frost hardiness, major changes take place within living cells of the tree. In general, cell sap concentration increases with freezing tolerance. The flow of water in and out of cells is facilitated by the increased permeability of the cell membranes and the cytoplasm, which changes from a translucent sol to an opaque gel state. These changes reflect the numerous biochemical reactions that occur in the cells during hardening and which have therefore attracted considerable attention. Numerous conflicting observations are available on the subject.

Many good correlations have been found between increases in frost hardiness and increases in chemical substances, such as sugars, proteins, amino acids, lipids and nucleic acids. However, these correlations rarely apply to these substances simultaneously, and frequently are considerably poorer during the dehardening period. These increases in substances result in a general protoplasmic augmentation during hardening (Siminovitch et al. 1967, 1968; Pomeroy et al. 1970). The increases in the various substances are frequently due to the accumulation of reserves for the winter but they also appear to serve as protective agents against freezing injury.

Most of the research concerned with the frost hardiness of higher plants has dealt with the biochemical changes that occur during the periods of frosthardening and dehardening. Almost all cellular constituents have at one time or another been analyzed with respect to a possible involvement in the frost hardiness process. In spite of all the research in this area there is little agreement on the significance of all these biochemical changes, as indicated by the many reviews on the subject (Alden and Hermann 1971, Heber and Santarius 1973, Levitt 1980). That this most extensively studied area of frost hardiness is the least understood is an indication of its complexity. Steponkus (1978) has discussed several good reasons for this dilemma. One of the reasons is that frost hardiness is a complex physiological process that is interdependent with many other physiological processes such as photosynthesis, respiration, transpiration, water and nutrient uptake and food reserve accumulation.

9.1.6 Frost Hardening Stages

The frost hardiness process in our native coniferous species occurs in two or three stages. The first stage occurs in early fall when the decreasing photoperiod becomes noticeable while the day temperatures are still relatively warm, but the nights are cool. The start of the first stage of hardening is associated with growth cessation, the initiation of terminal buds, the onset of dormancy and in the case of deciduous hardwoods, the onset of autumn coloration. During this initial hardening stage increases in frost hardiness are moderate. As the first hardening stage progresses, plants become increasingly responsive to temperatures near or just below the freezing point, which initiate the second stage of frost hardening. It is during this second stage that large increases in frost hardiness occur. The third stage of hardening is induced by temperatures of -15° to -50°C and only the extremely hardy species are able to attain this third stage (Sakai 1965) but this kind of hardiness is quickly lost (Weiser 1970).

Although the "stage" concept of frost hardiness development has been discussed by several investigators as being applicable to woody plants native to temperate climates (Tumanov and Krasavtsev 1959, Krasavtsev 1968, Weiser 1970, Glerum 1973b, 1976, Timmis and Worrall 1975) there is still no consensus on this concept among frost hardiness scientists. Levitt (1972, 1980) is not in agreement with the stage concept because it is possible to induce as many stages of hardening as desired by means of a graded series of hardening treatments. However, under natural hardening conditions, these stages are clearly noticeable.

Over an approximate 32-month period of frost hardiness testing (Fig. 1 and 2) with 3- to 4-year-old potted white pine (Pinus strobus L.), which were grown out-of-doors, the transition between the first and second stage of hardening occurred between mid and late October of every year (i.e. 1968 to 1971). The transition from the first to the second stage occurred at a hardiness level between -20° to -25°C and was clear in three of the four falls, being least evident in October
1968 (Fig. 2) because temperatures in the tall decreased more quickly in 1968 than in 1969, 1970 and 1971. The duration of the transition depends mainly on the external factor of temperature. The transitions in Figures 1 and 2 appear not as distinct as they could because the time scale is calibrated in months, while the transitions generally occur over a period of a few days to a few weeks.

Dehardening also appears to occur in two stages, but since dehardening in the spring occurs so rapidly, it is difficult to identify separate dehardening stages. In most instances, the transition from one stage of hardiness to the next will not be detected, unless the frequency of testing is increased from days to hours. In a study where seven coniferous species were tested, only one (Pinus resinosa Ait.) showed a distinct transition between two dehardening stages (Glerum 1973b).

9.1.7 Differential Frost Hardiness

Differences in frost hardiness within the same tree do occur. They occur between the various tissues, such as phloem, cambium and xylem, and also between various tissue components, such as needles, buds and bark. Reproductive parts are generally several degrees less hardy in winter than the apical meristem and the leaf primordia of vegetative buds (Larcher 1973). Furthermore, these differences change in relation to each other during the course of the year. Frost rings (Fig. 3) provide a good example of differential hardiness. At the time of freezing, the differentiating xylem is most sensitive (least hardy), the active cambium somewhat less and the phloem the least sensitive (Glerum and Farrar 1966). These differences in tissue hardiness make it extremely difficult to assess total tree damage and has led Larcher (1968) to suggest that frost hardiness testing of trees be standardized.

The greatest differences in frost hardiness within a tree occur between the roots and top

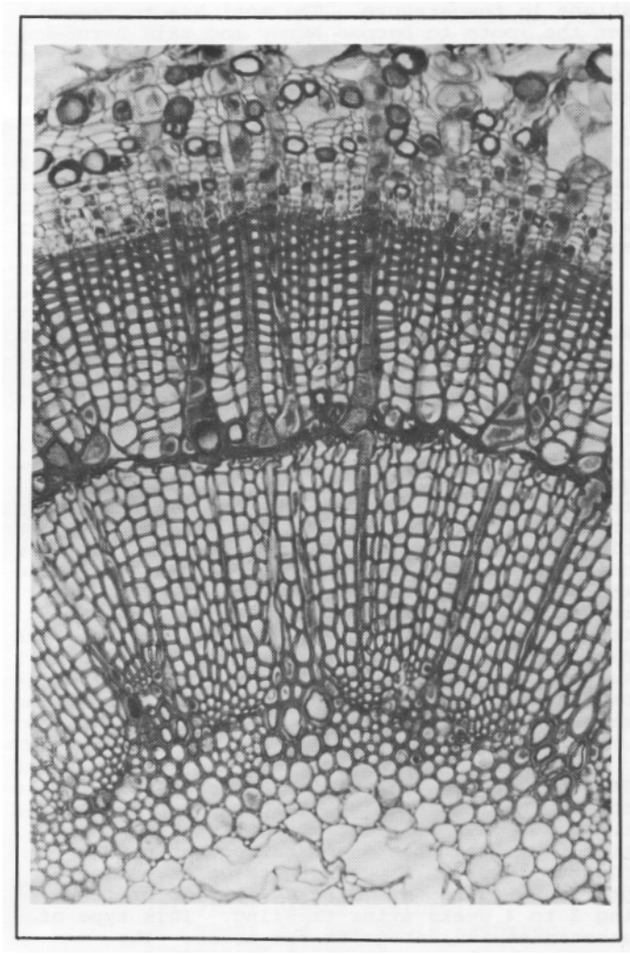

FIGURE 3 FROST RING IN THE XYLEM OF A PICEA GLAUCA STEM. THE DARK LINE IS THE LAYER OF CRUSHED CELLS, WHICH WERE IMMATURE TRACHEIDS IN VARIOUS STAGES OF DIFFERENTIATION BUT PREDOMINATELY CELL ENLARGEMENT AND SECONDARY WALL FORMATION. BELOW THIS LAYER ARE DEFORMED AND INCOMPLETELY LIGNIFIED TRACHEIDS BECAUSE THE FREEZING STOPPED THE LIGNIFICATION PROCESS. ABOVE THIS LAYER ARE SOME PARENCHYMA CELLS AND ABNORMAL TRACHEIDS AFTER WHICH THE REGULARITY OF THE RADIAL FILES IS RESUMED. NOTE THE ABSENCE OF ANY DAMAGE IN THE PHLOEM. (450X) (FROM GLERUM AND FARRAR 1966)

(i.e. the above-ground portion). The roots are significantly less hardy than the aerial parts of the tree by as much as 20 Celsius degrees (Pellett 1971, Steponkus et al. 1976).

The hardiness of the roots appears to be even more dependent on the environment it grows in (i.e. the soil) than the top, probably because the roots are buffered against drastic fluctuations in temperature. The soil has to freeze for the roots to become hardy and this hardiness is quickly lost when the soil thaws (Glerum 1961, unpubl. data). However, moderate increases in root hardiness generally occur prior to freeze up (Timmis 1984, pers. comm.). As soon as the frost is out of the ground in the spring, the roots begin elongating and have lost their hardiness, while the tops are still very hardy. In fact, the tops will not be completely dehardened for as much as another 8 weeks.

9.1.8 Methods for Evaluating Frost Injury

There are numerous methods for assessing frost injury. A good review has been provided by Timmis (1976a), and includes an evaluation of the most promising methods that can be used quickly and on a large scale. The injury tests, also called viability tests, are based on whether or not metabolic and enzyme functions have been impaired and whether or not cell membranes have been damaged resulting in the loss of the selective permeability of the injured protoplasts. The existence of so many tests, suggests that there is no single satisfactory 100% reliable test. It should also be kept in mind that most of these methods have been developed to meet specific study requirements.

It is desirable to use more than one method for evaluating freezing damage. With intact trees a good assessment method is a combination of visual damage and subsequent growth (Glerum 1973b). A popular damage assessment procedure is to determine the extent of browning 2 to 4 weeks after freezing. This type of assessment is inadequate for trees. The appropriate length of the injury assessment period after freezing depends on the phenological state of the tree at the time of testing. For example, when assessing damage outside in early spring (April) two weeks after freezing was too short and four weeks after was better, but in May two weeks after freezing was better than either one or four weeks. In September, 8 weeks after freezing was better than 4 weeks and in the winter the best assessment period was 12 weeks after freezing (Glerum 1973b). A modification of the browning test is when seedlings, after freezing, are placed in a growing environment, then a valid assessment can be made between 3 to 10 days.

On an operational basis tests of injury should be applicable on a large scale and fast. Tests such as vital staining, plasmolysis, and amino acid leaching to mention a few, probably will never be used on an operational basis. On the other hand, a detailed browning test, electrical impedance and electrical diffusate conductivity tests are already used on a semioperational to operational basis.

9.2 Applications

Frost hardiness testing consists of two parts: 1/ the subjecting of seedlings or parts thereof to freezing temperatures, and 2/ the subsequent assessment of freezing injury. For many years hardiness was determined by field survival, which required years of observation since test winters occur only about once in 10 years. In the last 50 years numerous types of freezing apparatus and procedures have been developed to get answers more quickly, so that now field trials are only part of the final frost hardiness test.

9.2.1 Freezing Tests

Freezing tests can be conducted in the field (i.e. in situ) with portable freezing units or in the laboratory, but the use of a freezing chamber in the laboratory is the most popular. Types of freezing apparatus range from units that can be taken into the field to freezing bars and freezing chambers that have precise programmable microprocessors as temperature controllers. A critical evaluation of the various freezing apparatus has been provided by Warrington and Rook (198U).

The best way to conduct freezing tests in a tree nursery is to use a freezing chamber with a cam operated temperature controller, where the cams are cut in accordance with the temperature cycle desired. The minimum temperature to which seedlings are exposed, will depend on the seasonal cycle of frost hardiness. Generally, a range of minimum temperatures are used that bracket the seedling's frost hardiness. The rate of freezing is important and should be between 2 and 6 degrees C/hr. Fast rates of freezing can be injurious and compound the frost injury. The duration of the minimum temperature is also important and should be 1 to 3 hours long. Long exposures, such as 24 hours and over, can increase injury. The rate of thawing can be much faster than the rate of freezing (up to 20degrees C/hr). Plant material should only be used once, as repeated freezing can be injurious leading to erroneously high degrees of injury. It is important that all tests are done in exactly the same way, otherwise the results will not be comparable.

Tests can be done on intact seedlings or parts thereof, usually shoots. When using intact seedlings, the roots should be insulated because the roots will be considerably less hardy than the shoots. Insulate with sawdust, vermiculite, dry peat moss or any other good insulator. Intact seedlings will provide a better approximation to the actual frost hardiness than detached shoots or needles. This is partially due to the differential frost hardiness mentioned earlier. Detached shoots generally show a greater degree of frost hardiness than the intact tree (Glerum 1973b, 1976), but there are some investigators who are not in agreement with this observation (Cannell and Sheppard 1982). However, good results can be obtained using either intact seedlings or just parts thereof.

There are sophisticated and expensive freezing chambers available that can go down to -70'C. For frost hardiness testing at a tree nursery, a freezing chamber that goes down to -40'C will suffice. The temperature distribution within the chamber should be uniform. In many instances differences in frost hardiness have been observed, which were really due to uneven temperature distribution. Therefore it is important that prior to frost hardiness testing, the freezing chamber has been tested for temperature distribution.

Freezing rates can be easily monitored with automatic temperature recorders. There are many types of temperature measuring devices available but we prefer the use of a multichannel thermocouple type of temperature recorder, which can measure from 6 to 24 separate points. Copper-constantan thermocouple wire is most useful because it can be easily made of various thickness and to various length and does not rust. Thermocouples also are useful to monitor tissue temperatures, which is desirable and the soil when using intact seedlings.

9.2.2 Freezing Injury Assessment Techniques

As mentioned earlier, there are many methods that can be used for evaluating frost injury but at present only two methods can be considered operational: 1/ the growth or browning test and 2/ the electrolytic conductivity method; while 3/ electrical impedance and 4/ electrical impedance ratio can be considered semi-operational.

9.2.2.1 Growth or browning test

A refinement of the browning technique is where tissues such as buds, needles and cambium are examined separately, when after the freezing test, they have been placed in a growing environment. This procedure, which is also called "the whole seedling assessment method", has been successfully used, operationally by the Industrial Forestry Association, Toledo, Washington for nearly 10 years and in various frost hardiness studies (Timmis 1976b, 1977; Blake et al. 1979).

With this method, normally eighty seedlings are dug out of the nursery and placed after root pruning, with their roots in test tubes containing water. Twenty seedlings are used for controls while 3 lots of 20 seedlings are subjected to three different freezing temperatures. After thawing the seedlings are placed up to their root collar in beakers of water and left in a growing environment for 3 to 10 days at which time the needles, buds and cambial area of the stem are examined for freezing injury. The colour of healthy tissue is fresh green but when the tissue is injured the colour changes, over time from fresh green to a drab olive green to brown. Frost damage to needles and buds becomes evident within 3 days after freezing but damage symptoms to cambial tissue require 7 to 10 days after freezing to develop. Buds are sliced longitudinally and the tissue inside is examined. The stems are scraped to expose the cambial layer for the full length of the stem. Needle and bud damage are rated on a scale of 0 to 10 while cambial damage is rated on a scale of 0 to 4. Up to 10 buds are checked if available. For cambial damage 1 = top 1/4 dead or girdled, 2 = top 1/2 dead or girdled, 3 = stem girdled in lower 1/4 and 4 = entire stem dead. Seedlings are subsequently rated for viability using a rating schedule such as the one presented in Table 1.

An example of a completed assessment, after freezing at three temperatures (-8, -10 and -12) is given in Table 2. To obtain LT50 or any other LT, such as LT10, the viability rating (K) is used as illustrated in Table 2. These values are plotted as shown in Figure 4 and the LT values are interpolated from the graph. It is important to note that the assessment procedure illustrated is primarily

TABLE 1. A VIABILITY RATING THAT IS USED IN THE FROST DAMAGE EVALUATION PROCEDURE FOR THE WHOLE SEEDLING ASSESSMENT METHOD.[a]

Viability rating	Damage scale		
	Needles	Buds	Stem
0 (economically viable)	0-10	0-8	0-1
0.5 (half kill)	0-10	0-5	0-2
	0-10	9	0-1
1.0 (kill)	0-10	9-10	2-4
	0-10	0-8	3-4
	0-10	10	0-2

[a]Courtesy of C.J. Sally Johnson, Seedling Quality Services, Centralia, WA 98531. See text for further details.

TABLE 2. FROST DAMAGE ASSESSMENT OF NEEDLES (N), BUDS (B) AND STEMS (S), USING A 0-10 SCALE FOR NEEDLES AND BUDS AND 0-4 SCALE FOR STEMS. SEEDLINGS ARE SUBSEQUENTLY RATED FOR VIABILITY (K).[a]

#	Control				-8°C				-10°C				-12°C			
	N	B	S	K	N	B	S	K	N	B	S	K	N	B	S	K
1	0	0	0	0	1	0	0	0	10	9	3	1	10	8	3	1
2	2	0	0	0	1	0	0	0	10	9	1	.5	10	10	3	1
3	1	0	0	0	3	0	0	0	9	9	0	.5	10	10	3	1
4	0	0	0	0	1	0	0	0	8	7	1	0	10	9	3	1
5	0	1	0	0	2	0	0	0	10	10	1	.5	10	10	4	1
6	0	0	0	0	3	0	0	0	9	9	0	.5	10	9	1	.5
7	0	0	0	0	2	0	0	0	8	7	0	0	10	9	1	.5
8	1	0	0	0	2	1	0	0	10	9	1	.5	10	10	2	1
9	1	0	0	0	3	0	0	0	10	9	1	.5	10	10	4	1
10	0	0	0	0	1	0	0	0	10	9	0	.5	10	9	2	1
11	0	0	0	0	6	9	0	.5	7	4	1	0	10	10	3	1
12	0	0	0	0	9	3	1	0	10	9	2	1	10	10	2	1
13	0	0	0	0	2	0	0	0	8	9	0	.5	10	10	3	1
14	6	9	0	.5	2	1	0	0	8	4	0	0	10	10	2	1
15	0	0	0	0	8	9	1	.5	10	9	1	.5	10	8	3	1
16	1	0	0	0	2	0	0	0	7	4	0	0	10	8	3	1
17	1	0	0	0	1	0	0	0	10	9	2	1	10	9	1	.5
18	0	0	0	0	7	9	0	.5	9	10	1	.5	10	9	3	1
19	0	0	0	0	2	0	0	0	9	10	1	.5	10	9	2	1
20	0	0	0	0	1	0	0	0	10	10	2	1	10	10	3	1
				.5				1.5				9.5				18.5
	$\frac{.5}{20}$x100=2.5%				$\frac{1.5}{20}$x100=7.5%				$\frac{9.5}{20}$x100=47.5%				$\frac{18.5}{20}$x100=92.5%			

[a]See table 1 for explanation.

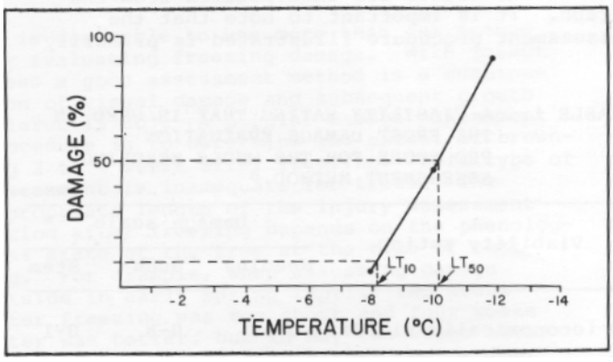

FIGURE 4. PERCENT FROST DAMAGE AT THREE FREEZING TEMPERATURES (-8-, -1U-, -12°C) AS DETERMINED WITH THE WHOLE SEEDLING ASSESSMENT METHOD (TABLE 2). LETHAL TEMPERATURES FOR ANY PERCENT DAMAGE CAN BE DETERMINED FROM THE GRAPH. LETHAL TEMPERATURES CAUSING 10 and 5U PERCENT DAMAGE (i.e. LT10 AND LT50) ARE SHOWN HERE.

for Douglas fir and that the emphasis is on economic viability. It other species are to be tested and the emphasis is to be on total viability this assessment procedure will require some adjustment.

Depending on the phenological stage of development of the seedling at time of freezing, a valid assessment can be made in 3 days, when freeze testing in early fall or late spring. but it can take up to 10 days before a valid assessment can be made when the freezing tests are conducted in mid-winter.

9.2.2.2 Electrolytic conductivity method

This method is based on the fact that when tissue is injured the site of injury is the cell membrane, which loses its selective permeability. Thus, upon injury the electrolytes that occur in the aqueous cellular cytoplasm move more freely and diffuse (leach) out of the tissue when it is placed in water. The severity of the injury is proportionate to the amount of electrolytes that diffuse out of the tissue. By comparing the conductivity of uninjured tissue diffusate with that of injured tissue, an estimate of the amount of injury can be made. This method, developed by Dexter et al. (1930, 1932) and further tested by Wilner (1962), has proven to be useful in a wide range of situations including many tree species. The technique was not strictly quantitative, because of variations in total electrolytes in different samples and was therefore refined by Flint et al. (1967). They proposed a scale where the unfrozen sample is given a value of zero and the totally killed sample a value of 100 and thus the release of electrolytes is expressed in percent. Flint et al. (1967) called this the "Index of Injury" (It), which by now has received considerable testing in forestry, in particular by S.J. Colombo of our Institute, who has found it to be an excellent technique for determining the frost hardiness of coniferous containerized seedlings. A manual has been prepared to assist nurserymen in guiding their stock so that it will be properly conditioned for overwintering (Colombo et al. 1984).

An outline of the procedure is presented here but a copy of the manual should be obtained, because it outlines each step that has to be taken towards the determination of the it value.

Step 1 Collect 45 shoot tips (2-3 cm long) randomly from seedlings of the same seed source. With 1-year-old container stock, use the terminal shoot, but with 2- to 3-year-old stock use top laterals.

Step 2 Fifteen shoot tips are to be used for the non-frozen control and are rinsed with distilled or deionized water.

Step 3 Shoot tips are immersed in distilled or deionized water and left at room temperature (2U-25"C) overnight. Shoots should remain totally immersed at all times. Aluminum foil or any inert substance can be used to prevent the shoots from floating. We are using capped jars in which 90 ml of water is sufficient to submerge shoots. It is important not to lose any water at any time during the entire process.

Step 4 Shake jar and measure conductivity with a conductivity meter. This gives the control electrical conductivity, i.e. EC control.

Step 5 Shoots are killed by placing shoots and capped jars in oven at 90'C for 2 hours.

Step 6 Killed shoots are left overnight at room temperature.

Step 7 Shake jar and measure conductivity of killed shoots, i.e. EC control killed. The relative conductivity of control sample is:

$$\text{RC control} = \frac{\text{EC control}}{\text{EC control killed}} \times 100$$

and is that proportion of the shoots total electrolytic content which is released without freezing.

Step 8 Thirty shoot tips to be frozen are rinsed with distilled or deionized water before jar is capped and placed in freezer where they are equilibrated to 5'C for 1 hour. Then temperature is decreased at a rate of 5 Celsius degrees per hour to the desired minimum test temperature. A minimum test temperature of -10'C is used for nonhardy tissue, but as frost hardiness develops minimum temperatures of -15'C to -30'C can be used. At present we test at both -l0'C and -15'C throughout the monitoring schedule.

Step 9 When test temperature has been reached the sample is removed and allowed to warm up (thaw) slowly overnight (i.e. by placing in a cooler).

Step 10 Sample is split into two replicates of 15 shoot tips each and immersed in distilled or deionized water, similarly to step 3.

Step 11 Capped jars are left overnight at room temperature to allow the electrolytes to diffuse into the water from the injured shoot tips.

Step 12 Shake jars and measure conductivity of the frozen shoot tips (i.e. EC frozen).

Step 13 Kill frozen shoot tips by placing shoots and capped jars in oven at 90'C for two hours after which they are left overnight at room temperature.

Step 14 Shake jar and measure conductivity of the killed frozen shoot tips (i.e. EC frozen killed).

The relative conductivity of the frozen sample (RC frozen) is:

$$\text{RC frozen} = \frac{\text{EC frozen}}{\text{EC frozen killed}} \times 100$$

and is that proportion of the shoot tips total electrolytic content, which is released due to the freezing injury.

Frost hardiness is expressed using the "Index of Injury" (It) which is calculated on a percentage basis as:

$$I_t = \frac{\text{RC frozen} - \text{RC control}}{1 - \frac{\text{RC control}}{100}}$$

where It is an expression of the amount of injury caused by freezing. When "I_t" is high the frost hardiness is low, and when "It" is low the frost hardiness is high. The results are plotted weekly and after a season of monitoring resemble those in Fig. 5.

9.2.2.3 Electrical impedance method

The electrical impedance method, which we have used extensively (Glerum 1973b, 1980b), involves the taking of an electrical impedance measurement with a 1 kHz impedance bridge (i.e. similar to a wheatstone bridge) before exposing seedlings or seedling parts to freezing temperatures and then another measurement after the freezer treatment has been completed. Steel pins, spaced 1 cm apart, make good electrodes.

Electrical impedance is influenced by some physical factors such as tissue size (i.e. diameter) and temperature as well as by the frequency of the current. These factors have to be taken into consideration when using this method. Impedance is high at low frequencies and low at high frequencies. Most impedance measurements are taken at a frequency of 1 kilohertz (1 kHz) although some investigators prefer lower frequencies. Impedance will be higher at lower temperatures (e.g. 5'C) than at higher temperatures (e.g. 20'C). Impedance should be measured at room temperature, which eliminates the need for temperature correction. We prefer to measure impedance at a temperature of around 20'C (+2'C). The tissue temperatures of 2- to 3-year-old seedlings adjust to room temperature within one hour when brought indoors. It is important that all impedance measurements before and after freezing are taken at tissue temperatures that are the same.

Seedlings with small stem diameters will have higher impedance readings than those with larger diameters. The influence of diameter becomes minimal at about 0.5 cm and above, consequently it is preferable to use stems of that diameter. Besides the strong diameter effect with diameters smaller than 0.2 cm, electrode damage will also start to influence the impedance readings and therefore it is preferred not to use stems with diameters smaller than 0.2 cm.

Injury has occurred when the after freezing electrical impedance reading is much lower (from 50 to 80%) than the impedance reading before freezing. If the after freezing impedance reading is either slightly higher, the same, or slightly lower than the before freezing reading, no injury has occurred. Reductions of only 20 to 50% in impedance between the before and after freezing readings are difficult to interpret, which is partly due to the differential hardiness phenomenon.

Impedance measurements should be taken on the stem just above the root collar, because if heavy damage occurs at that point, the seedling is definitely of no use to the nurseryman. At present, this technique is insufficiently developed to differentiate between light and heavy injury to make it operational.

9.2.2.4 Electrical impedance ratio method

The kilohertz (kHz)/megahertz (MHz) impedance ratio was developed as a result of the electrical impedance dependence on frequency. The impedance at 1 kHz decreases with increasing injury until the death of the tissue. On the other hand, at 1 MHz the impedance of both living and dead tissue is approximately the same. The 1 kHz and 1 MHz impedance for dead tissue are approximately the same. Therefore, a ratio of impedance at 1 kHz over 1 MHz was developed as a criterion of vitality or injury. This ratio comes close to 1.0 when injury is fatal and should be above 3.0 when tissue is healthy (i.e. no injury). The advantage of this method is that only after-treatment measurements are required, as compared to the 1 kHz impedance technique, which requires a measurement both before and after treatment. Other advantages are that no compensation is necessary for tissue temperatures between 5° and 25°C, nor for diameter effects. Although it is still advisable to use diameters of around 0.5 cm if at all possible, and preferably not smaller than 0.2 cm.

The disadvantage of this technique is similar to that of the 1 kHz impedance technique, in that there is a large undifferentiated area between heavy injury and no injury. Impedance ratios below 2.0 indicate heavy to fatal injury, while ratios of 3.0 and higher indicate no injury. Ratios between 2.0 and 3.0 sometimes indicate injury and sometimes not. The grey area in this technique is to a large extent also due to the differential hardiness phenomenon. This technique is used operationally on a limited scale.

9.2.3 Predictive abilities

The differential frost hardiness phenomenon that is particularly pronounced in trees, precludes that there will be one single frost killing temperature. Nevertheless, it is still desirable to have a simple quantitative expression of frost hardiness for trees. As a result the electrical methods for assessing injury have received considerable attention in the last two decades because they appear to be the most promising. Of these, electrical conductivity with the "Index of Injury" is at present the most reliable technique and is used operationally in Ontario. The other reliable technique is the browning test, called the whole seedling assessment method, where needles, buds and cambium are examined 3 to 10 days after freezing when placed in a growing environment. This technique has been successfully used operationally for nearly a decade on the west coast.

The electrical conductivity technique is now being used in concert with freezing tests and bud dissections to guide the Extended Greenhouse Culture technique for hardening spruce container stock (Colombo and Glerum 1984). A typical monitoring pattern of frost hardiness and bud development in black spruce container seedlings is presented in Fig. 5. Note that negative It values are possible and that sometimes seedlings frozen to -10°C have higher It values than those frozen to -15°C. At the time of early bud initiation (point A) when height growth has nearly stopped, there is a temporary increase in frost hardiness (probably due to avoidance, rather than tolerance.) As the rate of needle primordia initiation in the bud reaches a maximum, the level of hardiness decreases, but when bud development is completed (point B) high levels of frost hardiness are maintained. In this example, seedlings were considered ready to be moved outside by October 25.

The predictive abilities of the "Index of Injury" for moving container stock from the greenhouse to outside has been good. Prior to the development of the Extended Greenhouse Culture technique, container stock in northern Ontario was sown in early June and moved outside around mid-August for overwintering. Heavy damage to the spruce seedlings of up to 50% occurred during overwintering. With the Extended Greenhouse Culture, damage has been reduced to less than 1%.

The Extended Greenhouse Culture technique allows for good bud development, which requires temperatures of 20°C. When critical

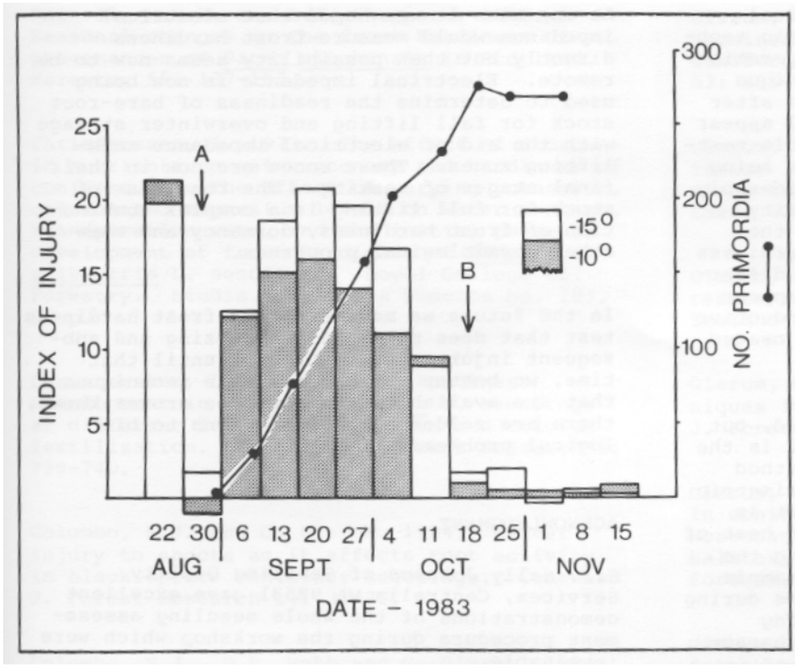

FIGURE 5

A TYPICAL PATTERN OF FROST HARDINESS AND BUD DEVELOPMENT FOR BLACK SPRUCE CONTAINER SEEDLINGS IN NORTHERN ONTARIO, CANADA. NOTE THAT MINOR DEVIATIONS OCCUR, SUCH AS NEGATIVE It VALUES (AUG 30; NOV 1) AND THAT SOMETIMES SEEDLINGS FROZEN TO -1U°C HAVE HIGHER It VALUES THAN THOSE FROZEN TO -15'C (AUG 22; OCT 11; NOV 1). AT THE TIME OF BUD INITIATION (A) THERE IS AN INCREASE IN FROST HARDINESS WHILE THE LEVEL OF HARDINESS DECREASES WHEN RATE OF NEEDLE PRIMORDIA FORMATION IS MAXIMUM. HIGH LEVELS OF HARDINESS OCCUR WHEN BUD DEVELOPMENT IS COMPLETED (8). SEEDLINGS WERE CONSIDERED READY TO BE MOVED OUTSIDE ON OCT 25. (COURTESY OF S.J. COLOMBO ET AL. 1984).

daylength (14.5 hrs), that triggers bud initiation is reached, the temperatures in the greenhouses are not allowed to go below 20 *C until bud development is near completion, at which time the heat in the greenhouses is switched off and the temperatures in the greenhouse are allowed to fluctuate with those outside to promote further frost hardiness development. The crop is moved outside after two successive weeks of low It values (Fig. 5) indicating high levels of frost hardiness. It is important to realize that once the crop is committed to the Extended Greenhouse Culture technique it has to be followed through to completion and cannot be aborted at any time because early removal would result in total crop failure.

A short-day culture technique is now being developed in which container stock is induced to initiate buds under an 8 hr-day. With this technique the crop will be ready to be moved outside earlier (i.e. early October) than with Extended Greenhouse Culture, thus saving both energy and time. However, the bud development and frost hardiness testing are also an integral part of this technique. In Ontario, no frost hardiness,testing is carried out for operational purposes on any other stock types.

Every method has its advantages and disadvantages and these two methods are no exception. The advantage of the whole seedling assessment procedure is that it deals with the entire seedling, while the electrical conductivity, as described, uses only seedling parts. Apparently, 1+U Douglas fir are particularly susceptible to frost kill at the ground line, near the root collar, which the whole seedling assessment method will detect, but the conductivity method, as described, will not. This phenomenon of frost susceptibility near the root collar has not been observed with our species and, therefore, we do not test the entire seedling. However, the conductivity method can be adapted for testing the entire seedling.

9.3 RECOMMENDATIONS

The only two methods for frost hardiness testing that have proven to be reliable on an operational scale are the freezing test, with associated 1/ electrical conductivity technique and index of injury or 2/ the whole seedling assessment procedure. These are the only procedures that can be recommended at this time. The electrical conductivity technique has proven to be useful with container stock and although this technique has not been tried on older bare-root seedlings, it should be applicable to those stock types as well. The detailed browning test appears to be well suited for 2- to 3-year-old bare-root stock as well as for one-year-old container stock.

It is important that when a new frost hardiness testing laboratory is established, a period of pre-testing is done before operational testing begins. During pre-testing the levels of I_t for non-hardy and hardy seedling shoots are determined for the new laboratory as these levels can differ significantly among laboratories. This applies to other techniques as well.

More research is needed on the electrical impedance and electrical impedance ratio techniques before they can be considered operational. Variations on the impedance technique such as using low frequency before and after freezing impedance ratios (Greer 1983) appear promising but still require considerable testing. Their greatest advantage besides being non-destructive is that they are considerably faster than the electrolytic conductivity method. But at this time, because of the confounding effects of differential hardiness the prospects of resolving impedance differences between light to medium damage appear remote. Our efforts might be more productive in exploring ways to shorten the time needed for determining index of Injury.

One method which has not been discussed, but has considerable operational potential is the differential thermal analysis (UTA) method (Wallner et al. 1982). UTA is a calorimetric method based on measuring the heat that is released as water freezes (i.e. latent heat of fusion). The method involves recording the differences in temperature between a sample twig piece and an oven-dried twig piece during freezing (Quamme et al. 1973). Freezing patterns in plant tissue pieces are characterized with two thermocouples connected in series. The first thermocouple is enclosed in a small chamber with the test tissue, while the other thermocouple, with the oven-dried tissue serves as a reference. A small aluminum block holds the thermocouples. The entire system is cooled at a known rate and temperature differences between the two thermocouples is continuously recorded. As the water freezes in the test sample a sharp increase in temperature occurs (first exotherm), while the reference temperature remains the same. Typically, a large initial peak is observed, due to extracellular ice formation which does not cause injury in hardy tissue. The freezing of supercooled water results in a second exotherm at progressively lower temperatures in hardening plants. This is called the low temperature exotherm (LTE) which is associated with injury.

This method has been predominantly used on xylem tissue, but it has also been used on many other tissues such as flower buds, vegetative buds and leaves, which all have their own characteristic LTE (Quamme et al. 1982). A close relationship is suggested between the xylem LTE and the ultimate survival of the plant (Burke et al.. 1976). The major drawback of DTA is that very hardy woody plants such as those native to the Boreal Forest of North America do not deep supercool and therefore do not exhibit an LTE (Burke et al. 1976, Sakai 1982). But is this applicable to all tissue components of these tree species? Furthermore, the temperature sensing devices might require refinement, because the supercooling in these species could be minimal and thus exhibit minute second exotherms. The DTA technique warrants further research.

At one time it was hoped that electrical impedance would measure frost hardiness directly but that possibility seems now to be remote. Electrical impedance is now being used to determine the readiness of bare-root stock for fall lifting and overwinter storage with the aid of electrical impedance safelifting zones. These zones are now in their final stages of testing. The readiness of stock for fall lifting is a complex combination of frost hardiness, dormancy and some other physiological processes.

In the future we may develop a frost hardiness test that does not involve freezing and subsequent injury assessment, but until that time, we better use the reliable techniques that are available. As any tree grower knows, there are seldom simple solutions to biological problems.

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