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FREEZING AND INJURY IN PLANTS

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M. J. Burke
Department of Horticulture, Colorado State University, Fort Collins, Colorado 80523

L. V. Gusta
Crop Development Centre, Crop Science Department, University of Saskatchewan, Saskatoon, Saskatchewan, Canada SFN OWO

H. A. Quamme
Agriculture Canada, Research Station, Harrow, Ontario, Canada NOR 1HO

C. J. Weiser
Department of Horticulture, Oregon State University, Corvallis, Oregon 97331

P. H. Li
Laboratory of Plant Hardiness, Department of Horticulture Science, University of Minnesota, St. Paul, Minnesota 55101

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INTRODUCTION

Freezing injury is a major cause of crop loss, and low temperature is reputedly the single most limiting factor to natural plant distribution (92). The stresses of late spring and early fall frosts, low midwinter minima, and rapid temperature changes cause various types of injury directly and indirectly associated with the freezing of water in plant tissues. These include crown kill in winter cereals, biennials, and herbaceous perennials; sunscald on thin-barked tree species; winter burn to evergreen foliage; blackheart and frost cracking in xylem of trees and shrubs; blossom kill; death of vegetative shoots in late maturing perennial species; death of buds and bark in plants which lose hardiness rapidly during transient warm spells in winter; and outright death of tender annuals.

While some species are always killed at the moment they freeze, others will tolerate extremely low temperatures (–196°C) in midwinter (104). The low temperature responses of most plants fall between these extremes, and freezing resistance may change markedly with season and stage of development. For example, hardy trees and shrubs which survive –196°C during winter dormancy may be killed at –3°C during active spring growth.

Acclimation is the term which describes the seasonal transition from the tender to the hardy condition in hardy species. The effects of shorter days, decreasing temperatures, and other factors which trigger or influence acclimation have been extensively studied (38, 94, 106, 118), as have the biochemical (32, 68, 69), physiological (23, 38, 39), and biophysical (8) changes which occur in plant cells and tissues during acclimation. Acclimation and the reverse process, deacclimation, will not be reviewed here. In short, some species acclimate extensively in response to environmental and endogenous factors; others acclimate only a few degrees; and some do not acclimate at all. In plants which acclimate, water content (degree of hydration) almost invariably decreases with increasing hardiness, and increases as plants deacclimate. Mature seeds and spores which contain little water are notably resistant to freezing stress.

The status of water in living tissues, its behavior during freezing, and its role in freezing injury are the subjects of this review. Our limited understanding of these topics restricts progress in attenuating or avoiding freezing damage via crop culture and management, physiological manipulation, or genetic improvement. We have attempted to write a review which will be useful to interested biologists and agriculturists who are not hardiness experts.

Plant freezing has been reviewed from several perspectives. In 1967 Olien (86) reviewed the thermodynamic aspects of freezing in herbaceous semihardy plants. Mazur (79) discussed the general physical aspects of freezing from a cryobiological viewpoint in 1969. Mayland & Cary (77) examined the biochemical and biophysical

aspects of freezing injury in 1970. Parker's 1963 review (92) emphasized ecological aspects of freezing injury in hardy forest species, as did the review by Alden & Hermann (3) in 1971. Weiser (126) discussed freezing in hardy woody plants in 1970. Russian workers, notably Tumanov & Krasavtsev (124), have also written extensively on freezing in woody species. In their classic book on low temperature effects in biology in 1940, Luyet & Gehenio (73) discussed some aspects of plant freezing. Levitt (64, 66) has thoroughly surveyed the world literature on freezing injury in plants in his books on environmental stresses in 1956 and 1972. Of particular interest is the extensive research on woody species by Siminovitch & Scarth (113) and Sakai (105), on winter cereal by Johansson (43), and on herbaceous tissues by Asahina (4).

In this review similarities and differences in freezing and freezing injury in different plant types are discussed. We have emphasized relatively new methods which can be used to characterize freezing processes in plants, and recent findings which cast doubt on some generally accepted views.

KINDS OF FREEZING AND FREEZING RESPONSES

Plants have evolved a variety of mechanisms for resisting cold temperatures. Some survive by avoiding rather than tolerating freezing, such as the annual plants with little or no frost resistance which survive by means of dehydrated seeds that are very hardy. Roots, crowns, and even tops of some herbaceous biennials and perennials survive because snow and soil moderate extremes of air temperature.

When plant cells freeze, ice forms either inside (intracellular) or outside (extracellular) of cell walls. Intracellular freezing is a cataclysmic event in the living protoplasm which disrupts the integrity of the cell causing death. It occurs in tender plants that lack the capacity to acclimate and in hardy plants before they acclimate. Some "deep supercooled" tissues in hardy plants also freeze intracellularly (24).

Plants which are tolerant to freezing generally undergo extracellular freezing. Extracellular ice formation occurs in the vicinity of the cell walls (66). Apparently ice can often accumulate in this site with few ill effects. Some agriculturally important herbaceous plants such as winter cereals can survive extracellular freezing to –25°C, and numerous hardy woody species can survive extracellular freezing at the temperature of liquid nitrogen (–196°C) when fully acclimated. In the next section we summarize what we think takes place during freezing in several groups of plants which differ in hardiness. The evidence and rationale for these interpretations is discussed in the ensuing sections.

There are various modes of survival for plants exposed to subfreezing temperatures in nature. Plants with high solute concentrations can avoid a few degrees of freezing because of their depressed freezing point. However, few species of agricultural importance have freezing point depressions of more than 4°C, and most fall in the range of 1 to 2°C. A few halophilic plants have freezing point depressions as low as –14°C.

Freezing point depression is the temperature at which melting occurs, but not necessarily the temperature at which plants will freeze because almost all plants

supercool several degrees regardless of their hardiness. Supercooling probably occurs because of a lack of nucleating substances necessary for ice initiation and ice growth barriers in some tissues. During controlled freezing, woody plant stems frequently supercool to –15°C. Under field conditions whole plants seldom supercool to this extent for reasons which will be discussed later. In mild climates the few degrees of protection afforded by supercooling and/or freezing point depressions could be a significant avoidance mechanism; survival of olive leaves has been attributed to their ability to supercool several degrees (60).

In the absence of nucleating substances pure water can supercool to –38°C. This temperature is called the homogenous nucleation point for pure water, i.e. the temperature at which spontaneous ice nucleation occurs in the absence of nucleating substances (22, 101). It has recently been found that some plant tissues (flower buds and living cells in the wood of most temperate zone trees) "deep supercool" to temperatures around –40°C even though adjacent cells in the same plant freeze at only a few degrees below 0°C. The lowest supercooling observed in xylem parenchyma cells has been –47°C (25). These very low supercooling points are thought to represent the homogeneous ice nucleation temperatures of the cellular solutions of the tissues involved. When nucleation does occur in cells which are "deep supercooled" freezing is probably intracellular and lethal.

Tender Plants with Little or No Freezing Tolerance

The first "killing frost" of autumn (–1° to –3°C) kills many tender annual plants such as corn, cucurbits, and beans, which have little if any capacity to acclimate. On the morning after such a frost, the injured foliage appears flaccid and water-soaked, cell membranes have lost their semipermeability, and intracellular compartmentalization is destroyed. During active periods of growth the leaves and new shoots of unacclimated hardy trees and shrubs are also injured by a –1° to –3°C frost. Supercooling and freezing point depression may afford slight protection in these cases, but when nucleation occurs in the plant, rapid intracellular freezing takes place causing irreparable damage; notably the destruction of membrane continuity.

Chilling injury in species of tropical origin occurs at temperatures well above freezing, usually between 0° and 14°C (74). This extreme example of low temperature injury in plants apparently results from temperature induced lipid structure transitions (99) which disrupt membrane proteins. Chilling injury is not a form of freezing injury, but lipid "phase" changes or other membrane structural changes below 0°C could be involved in freezing.

Plants with Limited Hardiness

Most plants which survive freezing temperatures do so by tolerating some ice formation in their tissues. Herbaceous plants such as spring wheat, peas, and potatoes, for example, withstand a few degrees of frost, and some (winter wheat, cabbage, and turf grasses) have the capacity to acclimate and may survive winter temperatures to about –25°C. Many of these species survive in regions where the minimum

air temperature falls below the tissue-killing temperature because regenerative growing points are located at or below the soil surface and thus they are protected by residual soil heat and the insulating properties of snow.

Soil and air environments are markedly different; the temperature in soil frozen to a depth of several feet under a few inches of snow may be –2° or –3°C when the air temperature is –25°C. Plants with growing points below the soil surface are particularly subject to indirect forms of winter injury such as flooding and soil frost heaving which can lift the crowns (110). Soil moisture can also influence hardiness and survival because it affects tissue hydration (88) and soil temperature. The temperature of moist soil fluctuates less than dry soil because of the buffering influence provided by the heat of fusion of soil water.

Herbaceous plants seldom supercool more than –1° or –2°C unless tissue moisture content is very low (88). In desiccated tissues ice crystals do not spread uniformly, and some groups of cells may supercool several degrees and then freeze suddenly (probably intracellularly) with injurious effects. In herbaceous plants that tolerate ice formation within their tissues, ice appears to propagate primarily through the extracellular spaces, and, as noted, lethal intracellular freezing is rare. It may occasionally occur in highly hydrated tissues when freezing is too rapid to permit migration of cellular water to extracellular ice crystallization sites.

Although plants with limited hardiness may avoid intracellular freezing, a temperature is reached at which dehydration stresses resulting from extracellular freezing cause death. Generally, hardier plants can survive with more of their water frozen than less hardy plants. The "deep supercooling" that has been found in some woody plant tissues has not been observed in herbaceous species.

Woody Plants which Deep Supercool

Most deciduous forest species and fruit tree cultivars avoid freezing in some, but usually not all, of their tissues by "deep supercooling" to temperatures as low as –40°C in midwinter (9, 25, 97). Apple is typical of such plants. During active summer growth apple tissues are very susceptible to freezing, and injury can occur at –2° to –3°C as a result of intracellular freezing. In the autumn and winter the tissues of apple trees acclimate and freezing becomes extracellular in some tissues while others supercool extensively. Acclimated bark and bud tissues freeze extracellularly after a few degrees of supercooling. Much of the water in the cambium, cortical, and phloem tissues of the living bark migrates to sites in the outer cortex where ice forms in large masses between cells (127). Considerable amounts of ice are accommodated in this region with little apparent damage. Ice masses also form between scales of dormant buds. Stem tissues of hardy apple cultivars which freeze in this manner survive slow freezing to –60°C in midwinter or immersion in liquid nitrogen if frozen slowly to –30°C first (105).

Xylem ray parenchyma cells supercool in such stems, however, and are killed at –40°C or above. The extent of "deep supercooling" in hardy plant tissues varies with the season and the hardiness of the plant, reaching a maximum in midwinter. It appears that "deep supercooling" is a survival mechanism for some plant tissues and

suggests that the cold acclimation process involves reduction or elimination of ice nucleating centers in cells of such tissues, development of effective barriers to nucleation by ice in or around adjacent cells, or both.

Death in xylem rays seems to be associated with a discrete and measurable freezing event in such plants, i.e. differential thermal analysis (DTA) measurements (Figure 1), revealing that heat is released at the temperature at which the xylem parenchyma cells are injured (Figure 1 and 2d). The DTA method will be discussed in a later section. In short, it is thought that the sudden release of heat observed at low temperature results from the intracellular freezing of supercooled water in living xylem cells and that it represents the killing point of these tissues (9).

This type of freezing pattern is typical of tree species native to the Eastern Deciduous Forest of North America, species with northern range limits extending only to latitudes where the minimum winter temperature reaches –40°C. Xylem rays

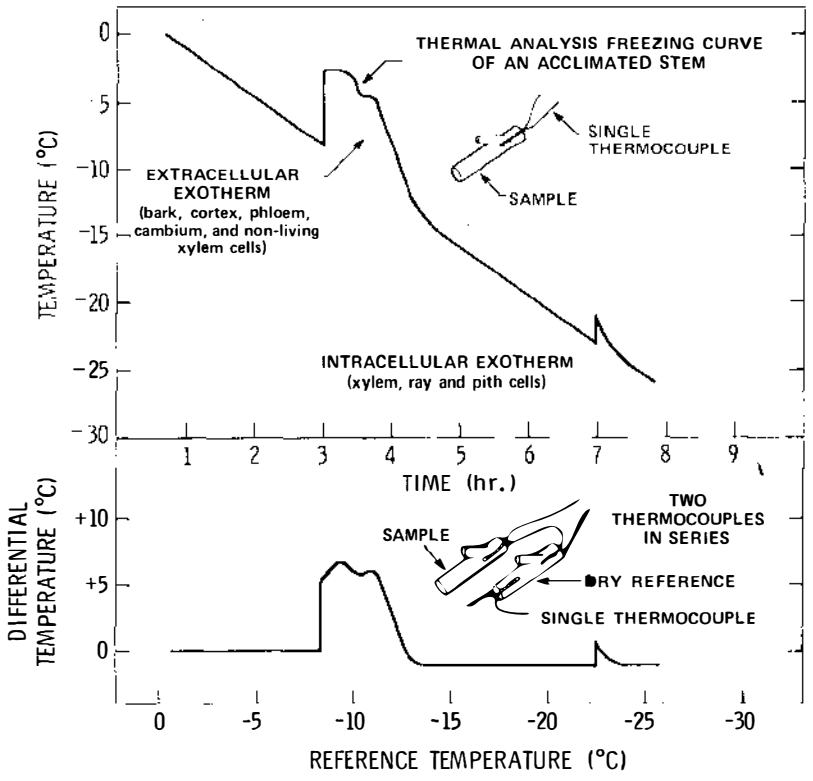

Figure 1 Comparison of thermal analysis and differential thermal analysis (DTA). In both experiments the sample is cooled over a period of time and the sample temperature or the differential temperature between the sample and a dry reference is monitored. The peaks or exotherms indicate freezing points.

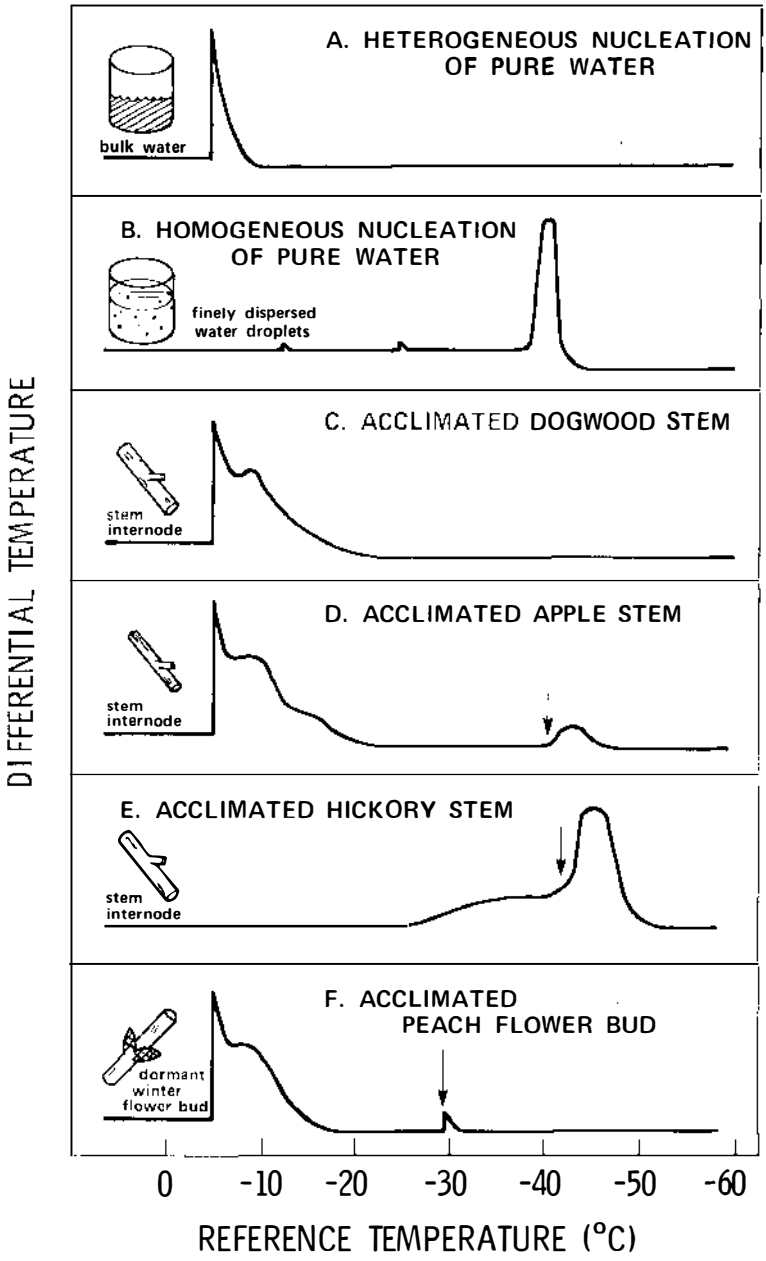

Figure 2 DTA of water and plant tissues.

are characteristically the least hardy stem tissue in midwinter, but they are often hardier (as much as 20°C) than the cambium, phloem, and cortical tissues of the bark in early autumn and late spring (97). Generally only part of the stem water supercools. One exception is shagbark hickory in which all the water in stem internodes supercools to about –40°C in midwinter (Figure 2e) (9).

Functional wood of trees and shrubs which transports water and nutrients consists largely of dead cells. Xylem ray parenchyma are apparently involved in lateral transport between young xylem sapwood and the cambium and living tissues of the bark, but their role is seldom considered to be vitally important to the survival of the plant. This gives rise to the question: Is death of xylem ray cells really important to the ultimate survival of a plant? The close relationship between the northern limits of the natural ranges of Eastern Deciduous Forest species and the probability of occurrence of minimum temperatures which will cause xylem injury (25) circumstantially suggests that the answer is yes. More extensive studies of blackheart injury in cultivated fruits such as apples and pears also support this conclusion. These fruit trees all exhibit deep supercooling in xylem, and blackheart injury is common in severe winters in northern production areas. In this type of winter injury the living xylem cells are killed, the wood becomes dark and discolored as a result of oxidation, and vessels become filled with gummy occlusions (20). Blackheart doesn't kill trees outright, but wood rotting organisms often invade injured trees and this combination of factors seriously limits productivity and tree longevity (11).

Deep supercooling also occurs in the dormant overwintering flower buds of some plants such as deciduous azalea, blueberries, apricots, peaches, cherries, and plums (Figure 2f). These plants also have "deep supercooling" xylem exotherms. Apple flower buds do not deep supercool although apple xylem does. In peach, for example, the flower primordia in dormant winter buds supercool to as low as –25°C. As in the case of xylem ray injury, the killing temperature of buds coincides with a sudden exotherm or release of heat (Figure 2f). These exotherms appear as buds begin to acclimate in the autumn, and shift to progressively lower temperatures as acclimation proceeds. They persist until flower buds expand in the spring. When flower buds begin expanding they supercool little and rapidly lose hardiness (98). Fully open flowers normally tolerate only –1° to –3°C of frost (5). Dormant winter buds of some hardy deciduous azaleas supercool in winter to temperatures as low as –41°C (26).

Woody Plants which Do Not Deep Supercool

Very hardy woody plants, such as those native to the Boreal Forest of North America, do not deep supercool. The extracellular freezing process is similar to that found in less hardy herbaceous plants, but ultimate hardiness is considerably greater. Ice formation begins somewhere in the plant after a few degrees of supercooling, and ice propagation proceeds through the extracellular spaces. This creates an extracellular vapor pressure deficit, and cell water is drawn from the protoplasm to the extracellular spaces where it freezes. In midwinter, many hardy woody plants survive the extreme dehydration that results when all of their freezable water crystallizes extracellularly. Generally, the hardier the plant the greater the capacity

of cells to tolerate dehydration. Paper birch, red-osier dogwood, willow, trembling aspen, and many other plants with this type of freezing pattern have natural ranges that extend to the arctic zones and survive experimental freezing to −196°C when they are fully acclimated (25). At such low temperatures, all the freezable water is frozen extracellularly (108). The unfreezable (bound) water fraction in winter stems of such species may amount to about 30% of the total water in the tissue (9). There is no apparent relationship between the amount of bound water and hardiness (9, 31).

METHODS FOR STUDYING FREEZING IN PLANTS

Some early determinations of ice formation in plants were indirect and assumed that plant tissue solutions froze as "ideal solutions" (2, 44, 63). Ideal freezing curves have been described (31) and can be calculated if cell solute concentrations, hydrostatic pressures, and the amounts of unfreezable or "bound" water are known. Initially freezing curves were predicted from estimates of cell solute concentration arrived at by measuring the freezing point depression of expressed plant sap or by plasmolytic measurements on intact cells (2, 63). Calorimetric measurements of the liquid water contents of tissues at two subfreezing temperatures provided a further refinement in freezing curve predictions (44). Some of the direct methods which have been used to study freezing are nuclear magnetic resonance (NMR), calorimetry, differential scanning calorimetry (DSC), DTA, phase contrast light microscopy, electrical resistance measurements, electrophoretic mobility and diffusion of dyes, dielectric constant measurement, and dilotometry.

Electrical resistance, electrophoretic dye mobility, and dye diffusion techniques have been developed primarily by Olien and co-workers in studies on model systems (89, 90), cereals (84), and some woody plants (18). These methods were designed to measure the fraction of unfrozen water in the extracellular spaces. They are based on the hypothesis that the hydrated extracellular spaces form a continuous liquid network throughout a plant; i.e. the mobility of dyes and ions is reduced in proportion to the reduction in extracellular liquid water during freezing.

Phase contrast light microscopy has been used to study freezing in cortical parenchyma cells of elder, birch, and apple bark (54). The refractive index of dehydrated cells was determined during freezing. These measurements were used to compute cell solute concentrations which is proportional to the degree of cellular dehydration. Calculations estimating the unfrozen water fraction from refractive index measurements agree well with calorimetric values.

Freezing has also been studied by measuring the expansion of ice in tissue via changes in its displacement (dilotometry) and specific gravity. Levitt (66) has reviewed these methods.

Nuclear Magnetic Resonance

In recent years NMR has been used to study water and water interactions in a wide variety of materials ranging from trees (8, 9), biopolymers and cells (6, 14, 59, 125), to silica and clay (16, 91, 93, 102, 103), and has been used to study liquid water in

frozen protein solutions (56–58), and to quantitatively measure the liquid water in frozen wheat-flour dough (121, 122), fish muscle (120), and plants (8). NMR is a type of spectroscopy which employs radio frequency light, and its utility for studying freezing in plant tissues depends on the large spectral differences between ice and liquid water. The spectrum for pure liquid water (a plot of radio frequency vs absorption intensity) is a single absorption line (Figure 3a). The width of this line is dependent on the state of the water. For example, ice and solids have such wide lines (30,000 to 100,000 Hertz) that they are not observed on conventional NMR spectrometers. Since line widths for liquid water in tissues are much narrower (usually one to several hundred Hertz) they show up clearly. The amount of liquid water is proportional to the area under the NMR line (Figure 3).

A stem section (0.5 cm long) placed in a refrigerated NMR sample chamber typically yields a single NMR line approximately 50 to 10,000 Hertz in width, depending on the temperature (Figure 3a). The line is narrowest above the freezing point and becomes progressively broader as freezing progresses. As the line broadens, the liquid water content—represented by the area under the line—becomes increasingly difficult to determine because of the low amplitude of the peak.

Pulse NMR is a particularly useful variation of NMR for studying plant samples (Figure 3b). It provides the same information as the NMR line method, i.e. continuous wave NMR, and the experimental configuration and sample requirements are similar. Detailed information on pulse NMR can be found in Farrar & Becker (21).

In pulse NMR studies, short radio frequency pulses (usually 90° or 180° pulses) are used to induce a transient magnetization in the sample. The signal following a 90° radio frequency pulse is called the free induction decay. It is recorded on an oscilloscope and the decay rate is related to the NMR line width (Figure 3b). The initial amplitude of the free induction decay is proportional to the area under the NMR line. Therefore, the initial amplitude is related to the liquid water content. The free induction decay for ice is so rapid that ice spectra are not observed in experiments designed to measure liquids (9). Pulse NMR methods have been used to characterize the freezing of winter cereal leaves (31), potato leaves (10), and woody stem tissues (8).

NMR methods provide one of the few ways to directly measure the liquid water content of partially frozen tissues. The curves obtained for winter cereal crowns (31) during freezing are similar to those predicted for ideal solutions. The shape of these curves suggests that there are two types of water in these tissues: a "free" or freezable water fraction which crystallizes progressively as the temperature is reduced, and a "bound" fraction of water that does not freeze at any temperature tested. The freezable water fraction behaves like a salt or sugar solution when it freezes, and the unfreezable fraction behaves like the bound water in frozen protein solutions (56–58).

It is widely thought that hardy plants contain more bound water than tender plants. Several attempts to correlate the quantity of unfreezable water in plant samples with their cold hardiness revealed no such relationship (8, 31). Dogwood stems and winter cereal all had 0.2 to 0.4 g of bound water/g dry tissue, and there was no correlation with hardiness (8, 31). This was true even though the hardiness

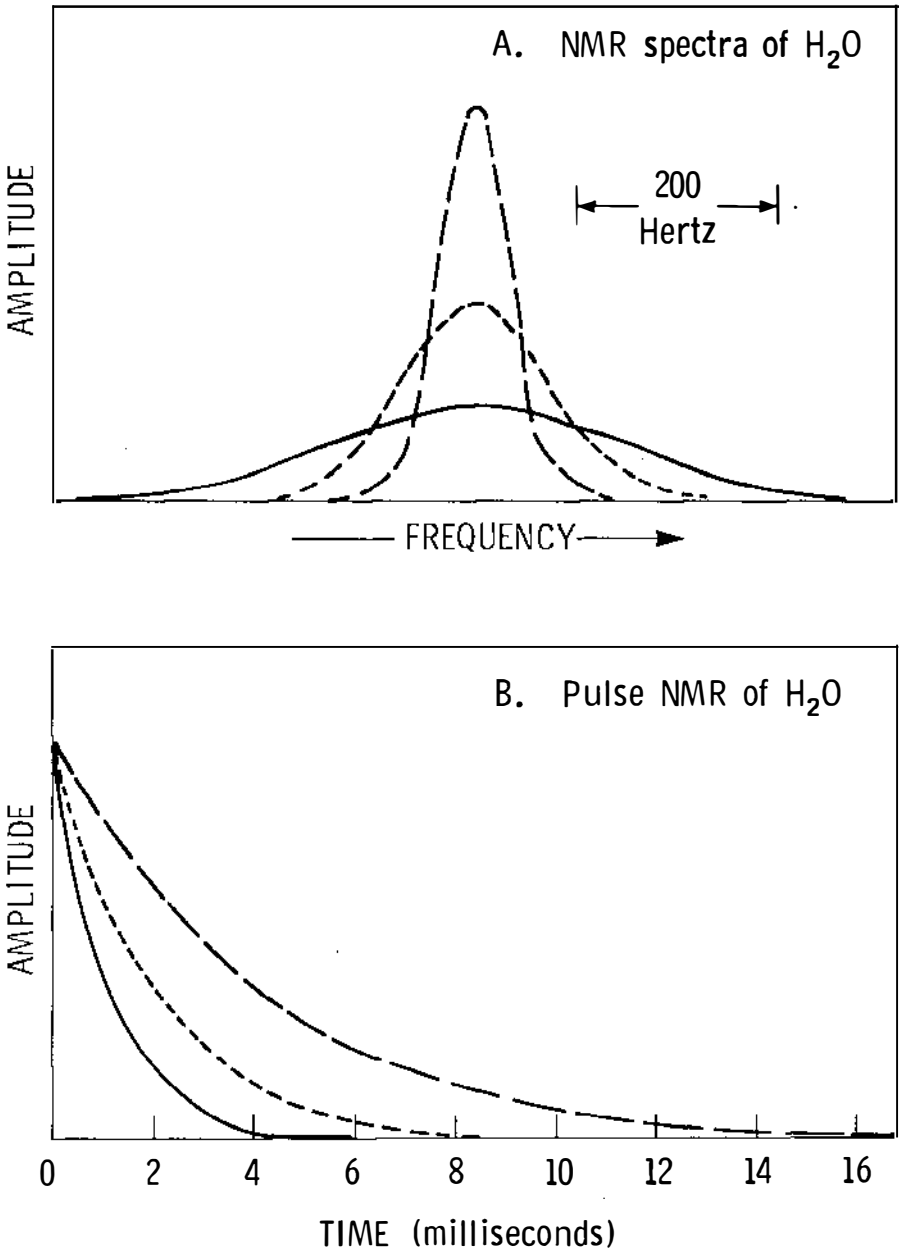

Figure 3 Nuclear magnetic resonance (NMR) spectra and pulse NMR of water.

of the tissues studied ranged from −3° to −196°C. Nonliving hydrated systems such as filter paper and proteins often contain more "bound" water than these plant samples (58, 89).

Calorimetry

Calorimetry basically involves the measurement of exothermic (heat releasing) and endothermic (heat consuming) events by recording relative temperature changes in a plant sample and a nonliving reference. Since the freezing of water is an exothermic reaction, temperature measurements of heat release in plant samples can be used to characterize freezing processes in plants. Similarly, water endotherms can be studied during thawing. The freezing and thawing of water are the predominant exothermic and endothermic events, respectively, in plant tissues.

There are basically three types of thermal analysis techniques which rely on these principles: (*a*) thermal analysis, (*b*) DTA, and (*c*) DSC. These methods are used primarily to determine the freezing and thawing points of tissue water, and, particularly DSC, to estimate the amount of water that freezes.

Thermal analysis is the simplest technique. It is particularly useful for determining the temperature at which exotherms occur during freezing by recording the temperature of a plant sample during freezing—e.g. inserting a thermocouple in a stem or bud and recording the temperature while the sample is frozen (Figure 1a).

DTA is a minor refinement to conventional thermal analysis as shown in Figure 1b. This is usually performed with two thermocouples in series, one in the reference and one in the sample. Data from the DTA are generally plotted as the temperature difference between sample and reference on the ordinate vs sample temperature, reference temperature, or time on the abscissa (Figure 1b). Several systems for DTA have been described for studying freezing in plants (26, 96).

DSC has been applied to freezing of plant tissues, and has employed two types of calorimeters: the Calvet calorimeter (43, 44, 51–53, 89) and the more conventional scanning calorimeter. These instruments provide the same information as thermal analysis or DTA, but they also quantify the amount of water that freezes or thaws between two experimental temperatures. Unlike other thermal analysis methods, which measure temperature or temperature difference, the differential scanning calorimeter measures differences in heat evolution or absorption between the sample and reference during cooling or warming. The amount of water which is frozen is determined by measuring the heat evolved during cooling, or absorbed during warming, and by making calculations based on the heat of fusion of water and the heat capacities of ice and liquid water.

A major weakness in using calorimetric measurements for determining liquid water content is in choosing the correct heat of fusion and, to a lesser extent, heat capacity for tissue water. When most of the water in a sample is still liquid it has a heat of fusion near that of pure water (79 cal/g). However, the heat of fusion may be considerably less at low subfreezing temperatures, where a larger fraction of the water is frozen (8, 89). Such unfrozen water will have a high solute concentration and will be at or near interfaces with macromolecules. In fact, NMR evidence

suggests that a fraction of tissue water is bound in some way to macromolecular structures. To freeze "bound" water, it is necessary to unbind water molecules before they freeze; the correct heat of fusion is, therefore, 79 cal/g minus the heat of binding. In many cellulose and protein systems, heats of binding may be as high as the heat of fusion so the heat of fusion for "bound" water may be zero or at least significantly lower than that of pure water (116). For example, water freezing in cellulose at −1°C has a heat of fusion of 12 cal/g. At −10°C the heat of fusion is 0 cal/g (89). Many calorimetric studies have not taken this into account.

Calorimetric methods are extremely sensitive to the freezing of minute quantities of water, and the thermal and DTA methods require only simple equipment. The disadvantage of these techniques is that it is difficult to quantify the amount of freezing that takes place.

An important recent application of thermal analysis and DTA has been detection of "deep supercooling" in some plant tissues previously described (Figure 2). This finding will be useful in testing the efficacy of chemical and cultural means of increasing hardiness and in providing plant breeders with selection criteria because deep supercooling of certain key tissues limits plant survival and crop productivity.

Hardy North American species such as *Populus tremuloides,* which range into the arctic and subarctic regions where the temperature frequently falls below −40°C, do not have low temperature exotherms (Figure 4). Species such as *Quercus rubra,* which have low temperature exotherms, are native to parts of the United States and southern Canada (Figure 4). The northern limits of their ranges coincide closely

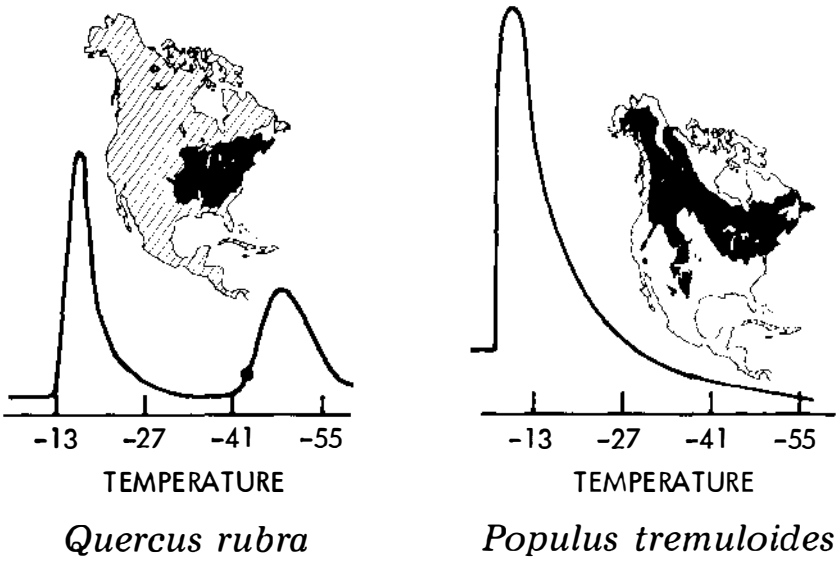

Figure 4 DTAs and geographic ranges of two species.

with the –40°C average annual minimum temperature isotherm for North America (Figure 4). This is also true for 28 other North American species tested (25).

It seems likely that the capacity to supercool is an adaptive mechanism in the xylem of certain deciduous species for avoiding the effects of freezing, but species which do not deep supercool survive lower temperatures. Specified wood or bud tissues which supercool often do not survive temperatures as low as tissues in the same plant which do not supercool. The adaptive advantage of deep supercooling, if any, is unclear. Interestingly, supercooling is found in deciduous trees with hard woods. It has been hypothesized that wood stiffness does not allow extracellular freezing, and collapse of the ray parenchyma is not possible. Therefore the only mechanism of survival in the ray parenchyma cells is deep supercooling (9).

FACTORS AFFECTING THE FREEZING PROCESS

Freezing Point Depression

As previously pointed out, freezing point depression itself does not appear to be an important means of freezing avoidance in plants. It lowers the freezing point of cell water and changes the freezing curve. In plants with limited hardiness, however, it could be significant. It has been suggested that cold acclimation of winter wheats is partially due to an increase in solute concentrations during hardening (43, 44). In plants with some tolerance of freeze-induced dehydration, a scheme has been outlined suggesting a relationship between freezing point depression and hardiness (66). There appeared to be reasonably good correlations in some tissues like wheat and rye leaves (43) but not in others like *C. stolonifera* stem, wheat crowns, and potato leaves (8, 10, 31).

Supercooling and Ice Nucleation

Water can remain in a supercooled state in plants if there is no external ice nucleation, and/or if the temperature does not fall below the homogeneous nucleation temperature. External ice such as hoar frost or soil ice can readily nucleate plants via entry sites such as stomates, lenticles, and wounds. Once initiated, ice grows rapidly through the plant from one or more nucleation sites. Ice grows most rapidly through the vascular tissues. The extent of initial supercooling influences the number and distribution of nucleation sites. Several nucleation sites may arise simultaneously if there is considerable supercooling or if there are a large number of favorable sites for nucleation by external ice. Since spontaneous nucleation is a chance event, large plants or plant samples are less likely to supercool extensively than small ones. Mulberry leaves, for example, supercool less when they are attached to the stem than when they are detached (49). Short spruce needles supercooled to lower temperatures than long ones because longer needles have more external nucleation sites and more water subject to chance spontaneous nucleation (47, 111). The location and distribution of external nucleation sites in boxwood (*Buxus*) leaves varied with age and maturity; older leaves were less likely to supercool (45). In many plants the likelihood of nucleation (from external ice) appears

to be dependent on morphological features of the plant rather than the presence of nucleating compounds suspended in water (46), e.g. a thick cuticle, if continuous, may be quite an effective barrier to seeding by external ice (114).

Nucleation and the extent of supercooling are important considerations in conducting tests on detached plant parts. Small excised plant samples tend to supercool more than whole plants for the reasons mentioned and may lead to erroneous conclusions when results from controlled freezing tests are extrapolated to the field. Freezing in a sample which is substantially supercooled is rapid, and may be more injurious than slower (equilibrium) freezing in a sample which has supercooled little. Excised samples are usually artificially seeded with ice crystals in various ways to circumvent this problem.

Deep supercooling observed in some woody plant tissues likely involves supercooling of an aqueous fraction which is effectively isolated from seeding by surrounding ice, and which is divided into small compartments. This may be analagous to the extensive supercooling observed in finely dispersed particles of pure water where homogeneous or heterogeneous ice nucleation in one particle (compartment) is isolated and confined to a small fraction of the total water (101). The majority of compartmentalized pure water then freezes at –38°C where pure water spontaneously nucleates. The presence of solutes and solvents in water can further depress the spontaneous nucleation point. The deep supercooling exotherm of shagbark hickory stems has been lowered experimentally by adding solutes (9).

The nucleation point appears constant in acclimated plants which deep supercool. At a given sampling date it consistently occurs at a certain temperature and is little affected by freezing rate (9). Equilibration of this supercooled water does not take place even under the considerable vapor pressure deficit at –35°C. In such deep supercooled systems the very slow freezing rate observed is due to ice nucleations and is of the order of magnitude of weeks or years at –35°C (9). In effect this supercooled fraction of tissue water is stable and present throughout the winter at temperatures below freezing, but above the homogeneous nucleation temperature of the supercooled fraction.

In xylem parenchyma and flower bud tissues, it is postulated that water is isolated in the tissues by barriers which prevent ice penetration from adjacent frozen tissues (9, 95), like the zone of relatively undifferentiated cells between the unfrozen flower primordia in the bud and the frozen stem tissues.

At the homogeneous nucleation temperature, however, freezing is rapid and almost certainly intracellular in deep supercooled tissues, causing death. It has been proposed (26) that barriers to ice propagation may involve fine microcapillaries of the cell wall; microcapillaries so small that they prevent ice seeding through the cell wall. An alternate hypothesis (26) is that antinucleating chemicals are present in protoplasm which prevent cellular nucleation. In any event, the deep supercooling phenomenon hinges on some structural feature because finely ground powders of tissues and tissue sectioned at 0.5 mm thickness do not deep supercool (9, 98). The structural feature does not directly involve the plasmalemma or protoplasm because supercooling can be demonstrated even in tissues killed by steam or chloroform treatments or oven drying and rehydration (98).

Ice Propagation and Growth

Once freezing is initiated, water molecules migrate and freeze at the surface. During much of the slow extracellular freezing, water becomes frozen in large ice masses at specific sites which accomodate ice with little damage to the plant. This type of localized freezing is apparent in the cortical tissues of the bark of hardy woody plants (127), in mesophyll of boxwood leaves (35), and in basal regions of flower and leaf bud scales (19, 127). The different patterns of water migration and ice formation observed in plants and plant tissues is probably related to their freezing resistance.

Electrophoretic methods indicate that water in cherry stems migrates to nucleating centers where ice grows unopposed. In azalea stems, which are less hardy than cherry, ice distribution is more diffuse and disruptive (18). In azalea flower buds, injury occurred at the base of the bud where large ice masses formed. The amount of injury appeared to be related to the amount of ice, which in turn was related to water content (71, 72).

Freezing rate influences patterns of ice growth and crystal size; fast freezing generally results in many small crystals, while fewer large crystals form when freezing is slow. Rapid freezing does not permit water migration to favored sites for ice crystal growth, and mechanical freezing damage can result. Furthermore, if freezing is very rapid, lethal intracellular freezing may occur (86). Single cells or small clusters of cells can survive –196°C if they are cooled and warmed so rapidly (by plunging into liquid nitrogen followed by rapid warming) that the small ice crystals formed are not disruptive (107).

Freezing and Thawing Rates

Rapid experimental freezing or freezing that follows deep supercooling of some tissues in nature generally results in intracellular ice formation. The importance of slow freezing rates on survival has been demonstrated (86). Most of the freezable water in a plant crystallizes between 0° and –10°C, and at slow cooling rates ice grows extracellularly at preferred sites which can accommodate growing ice crystals (19, 35, 127). When freezing was initiated at –2.6°C in winter cereals, the length of time for freezing to reach equilibrium at this temperature varied from 60 min in tender types to 30 min in hardier cultivars (Gusta, unpublished). Several investigators have shown that injury increases with the length of exposure to cold as the lethal temperature is approached (1, 29, 30, 40, 100). Hardy winter wheat was relatively unaffected by a cold treatment of –16°C for 1 hr, whereas all the plants were killed after 120 hr at –10°C. Repeated freezing and thawing also has an amplifying effect on injury (86). Plants may be slightly injured when subjected to a low nonlethal temperature but killed by subsequent cold treatments, e.g. fully acclimated hardy winter wheat withstood slow freeze to –19°C but was killed at –12°C after two thawing and refreezing cycles (Gusta, unpublished). The reasons for this are not known, but it may be related to "pools" of bulk water created when large ice masses are thawed. This may be resolved by varying the time (for water re-equilibration within the plant) between thawing and freezing cycles.

Tissue Hydration

A slight difference in moisture content of hardy tissues such as winter cereal crowns has been shown to have a significant influence on the freezing process and on cold hardiness (13, 41, 77, 81, 84). The optimum moisture content for overwintering cereal crowns has been reported to be about 65% (81). At higher moisture contents, most of the water freezes rapidly at a single freezing point, and the resulting ice crystals cause disruption of the tissue. Roots of most plants contain more water than crown or stem, and are killed at warmer temperatures (84). Partial dehydration of woody stems can increase their hardiness by as much as 7°C (67), while water in dehydrated cereal crowns may supercool and freeze intracellularly in isolated pockets.

Cell Walls and Membranes as Freezing Barriers

Cell wall polymers interfere with the freezing process by interacting with ice (85, 87) and modifying the shape of ice that forms. The interference does not inhibit freezing, but modifies the structure of the ice crystal formed and determines where ice will form. Small and imperfect ice crystals are formed which are considered noninjurious to the cell. Cell walls and membranes may also restrict the movement of water molecules to the growing ice front (40, 41, 65, 83, 113) or act as barriers which impede the advance of an ice front.

The plasmalemma is a major barrier to ice crystal growth into the cell (65, 78, 88, 109). It is also the major barrier controlling water permeability. During cold acclimation of some tissues, membrane lipids become less saturated (17, 55, 75) and more permeable to water (55, 66). This could alter freezing patterns and reduce the chances of intracellular freezing (41, 65, 86). In potato, however, no differences in water permeability were found between hardy and tender selections and species (119).

MECHANISMS OF CELLULAR INJURY

Injury Resulting from Intracellular Freezing

Unfortunately, the mechanisms of freezing damage to plants are poorly understood. Intracellular freezing occurs suddenly as cells flash freeze, cell by cell, with thousands of tiny ice crystals forming throughout the protoplast and vacuole (4). Cells in which ice crystals are visible through a light microscope are almost always killed (4). Injury probably results from the cataclysmic mechanical stresses and dehydration imposed on macromolecular cellular structures by ice. Membrane destruction is one of the most readily apparent manifestations of intracellular freezing but may be only one of a myriad of cellular sites of injury. Enzymes loosed by the breakdown of cellular compartmentalization rapidly wreak havoc in the injured tissue.

In hardy cells frozen at less than 1°C per hour, intracellular freezing is not thought to occur, but tissues which deep supercool are probably an exception since the freezing in such tissues is very rapid even if cooling rates were slow. The death

of supercooled tissues at the moment of freezing is, almost surely, the result of intracellular freezing. Light microscopic studies indicate that ice is present in ray parenchyma cells of shagbark hickory after they have frozen (9).

Injury Resulting from Extracellular Freezing

Hardiness in most plants appears to be related to tolerance of extracellular freezing (66). In effect this is a form of drought tolerance because removal of water from cells to extracellular ice imposes a considerable desiccation stress on the protoplasm. In many cases it is apparent, however, that desiccation alone is not the cause of freezing injury. Equivalent desiccation stress (from partial drying of samples) is also considerably less damaging when ice is formed in the plant than when it is not (119). Hence, damage caused by freezing cannot be attributed solely to desiccation but, in part, to the presence of ice in the tissue or other direct effects of low temperature such as the lipid phase separations in chilling injury or, as is more likely, by interaction of such factors.

Most of the current hypotheses proposed to explain freezing injury in one way or another suggest that dehydration of plant cells during freezing plays a primary role in the events which lead to death. These hypotheses include the sulfhydryl-disulfide hypothesis (65), the protein water shell hypothesis (36), the salting-out hypothesis (80), and the vital water hypothesis (126). A common denominator to all of these theories is the role that proteins play in cell structure and function. It has been shown that the membrane of most frost-injured cells are damaged (66) and that membrane-bound proteins may be involved (36, 66). There is direct evidence of loss in protein solubility (34, 61, 70, 123), and protein dissociation into subunits (28, 33, 48, 50) has been reported to occur following freezing. Loss in enzyme activity due to dehydration is also shown in cases of water stress (15, 117). Cyclic freezing and thawing of hydrated proteins increases denaturation in vitro (62). Glycerol, sucrose, dimethyl sulfoxide, polyvinylpyrolidone, ethylene glycol, and other cryoprotective agents have been shown to inhibit protein denaturation during freezing (12, 66, 115).

Protein denaturation during freezing and thawing has been attributed to changed pH (12, 34, 61), increased salt concentration (115), oxidation of sulfhydryl groups (48), protein concentration (12), and loss of water which maintains essential conformation (70, 76). There is evidence that the membrane is the primary site of desiccation injury (30, 37, 41, 112, 128). Uncoupling of phosphorylation in thylakoid membranes during freezing is accentuated by increased concentrations of solutes which reach toxic level during freezing (36, 37). One or more of these desiccation effects could result in cellular injury and death. Much needs to be resolved before the picture is complete.

On a macro scale large ice masses cause shearing of tissues as in the vascular tissues of wheat crowns (88) and azalea flower buds (27) and frost cracks produced by ice in developing pear fruitlets (82). Little is known about ice effects at the cellular level, but tearing and disruption likely result as ice crystals grow and the desiccating protoplasts shrink. Protoplasts of hardy cells are more elastic (66).

Molecular level temperature effects are also likely. Olien (86) discusses low temperature-induced changes that could take place in membranes. In addition to lipid phase separations previously mentioned (99), lipoprotein membranes at low temperature are subject to fracture along hydrophobic regions due to weakening of intramolecular hydrophobic bonds at low temperature (42). Protein stability per se is also affected by low temperature alone (7).

SUMMARY

Freezing avoidance and tolerance mechanisms have been described in different plant types and tissues. Some plants and tissues are killed at the moment of freezing while others survive crystallization of all their freezable water; some supercool only a few degrees while in others "deep supercooling" (–20° to –45°C) is apparently a common freezing avoidance mechanism; living bark and cambium cells in a hardy twig may be 20°C hardier than adjacent xylem ray parenchyma cells on a given day in winter; massive membrane destruction apparently causes immediate death in crowns of winter cereals or bark tissues of trees when they are in the tender state, but destruction and other manifestations of death become apparent only after several days when they are acclimated.

The array of freezing processes and plant responses to freezing seems complex and confusing. Some similarities, even among diverse plant species and tissues, provide the basis, however, for some unifying concepts. Intracellular freezing in nature is probably invariably lethal. "Deep supercooling" is a common and effective freezing avoidance mechanism in tissues such as xylem ray parenchyma cells and dormant flower buds of many species native to climatic zones where minimum temperatures seldom fall below –40°C.

In woody and herbaceous plants which do not deep supercool (e.g. winter cereals, potato, and very hardy trees and shrubs), NMR patterns of water freezing and thawing are very similar. Thawing is essentially the reverse of the freezing pattern except that deep supercooled water thaws near 0°C, not at the temperature it froze. In effect, water in plants freezes and thaws essentially as an ideal salt solution. Unfortunately, increasing hardiness is not simply a matter of increasing cell solute concentration as has been suggested, and the amount of unfreezable (bound) water in plants does not appear to bear any relationship to hardiness. The difference between hardy and tender plants which survive freezing to some extent can be stated simply: hardy plants survive when more of their water is frozen than tender plants.

Relatively recent applications of calorimetric, NMR, and DTA techniques have elucidated several aspects of plant freezing processes. While no simple answers have been found, the prospects for further resolution of freezing injury and tolerance are encouraging.

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