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Impacts of Freezing on Long-Distance Transport in Woody Plants

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Freezing temperatures can cause lethal injuries in living plant tissues and are a major factor limiting the long distance transport of water in the xylem and phloem. The ability of different species to avoid or tolerate freezing stress through various mechanisms can go a long way in explaining species-geographic distributions (Parker, 1963; George *et al.*, 1974; Burke *et al.*, 1976). Plant species that live in freezing climates have three options for survival: (1) die, leaving a protected seed bank behind, (2) remain active, or (3) become dormant. While the first strategy is used by annual herbaceous species, long-lived woody species that leave their above-ground biomass in place require mechanisms that allow persistence during winter.

Plants that remain active during winter are subject to freezing of living and nonliving tissues, including the vascular system. In living tissues, freezing can cause intracellular ice formation, which can kill the cells, or extracellular ice formation, which may protect the cells from damage due to ice crystals (Kuroda *et al.*, 1997). If extracellular freezing occurs, cellular dehydration becomes the main problem, although mechanical damage to cell membranes may also occur (Fujikawa and Kuroda, 2000). Within the nonliving conduits of the xylem, freezing temperatures are likely to cause cavitation resulting in losses of xylem function, depending on the size of the conduits as well as the freezing temperature and tension in the xylem. Phloem transport is also limited by cold temperatures as sap becomes more viscous and as sink strength and rates of phloem unloading decline, slowing the bulk flow of phloem sap. To remain active in winter, therefore, plants must prevent intracellular freezing in their living tissues, including in the phloem system, and maintain some xylem function, even if the soil is frozen. Plants that become dormant during winter discard their most vulnerable living tissues, but still need to prevent intracellular freezing in stems and roots, and must be able to recover xylem and phloem function in the spring.

The potentially lethal stresses caused by freezing and low temperatures and the strategies woody plants use to survive these stresses are the subject of this chapter. In the first part of the chapter, I discuss the primary mechanisms plants use to control freezing dynamics and reduce freezing injury in living tissues, as well as the physiological transformations that take place during cold acclimation. In the second part of the chapter, I focus on the limitations to long distance transport in the xylem and phloem and how the vascular systems of woody plants have adapted.

Survival of Living Tissues at Low Temperatures

In plants, widespread death of living cells caused by intracellular freezing can be prevented by structural features and chemical properties that lower the freezing point of water and direct where freezing occurs in the plant. The freezing point of water can be lowered by several different factors, including increased osmotic concentration (Fig. 19.1A), the absence of ice nucleators, the presence of hydrophilic surfaces and macromolecules that lower the potential energy of water, and by increased viscosity, which delays ice formation (Wolfe *et al.*, 2002). Water often occurs in finely divided volumes in plants so that the freezing temperature of water can vary substantially across different tissues and even adjacent cells, giving plants some control over freezing dynamics. Resistance to freezing in plants is based either on tolerance to extracellular ice formation, which causes cell dehydration, or on avoidance of freezing, particularly through supercooling.

Extracellular Freezing and Dehydration Tolerance

In most cold-tolerant woody species, extracellular freezing is an important mechanism for preventing intracellular freezing (Kuroda *et al.*, 1997; Fujikawa and Kuroda, 2000). As temperatures drop, extracellular ice is formed on the surface of the cell wall, in lumens of nonliving fibers or vessels or in the extracellular spaces (Guy, 1990; Ameglio *et al.*, 2001b). The formation of ice crystals lowers the water potential because ice has a lower chemical potential than liquid water corresponding to approximately −1 MPa per ˚C (Fig. 19.1B) (Hansen and Beck, 1988). Water molecules from inside the cell are drawn to the ice crystal surface outside the cell and freeze. As liquid water moves out of the cell, the concentration of dissolved solutes inside the cells increases. The increase in osmotic concentration lowers the entropy of the water molecules and depresses the freezing point by 1.86 ˚C per mole of solute dissolved per kg of water (Fig. 19.1B) (Chang, 1991). The redistribution of water from inside cells to extracellular ice masses causes cells to shrink in volume and has large effects on protoplas-

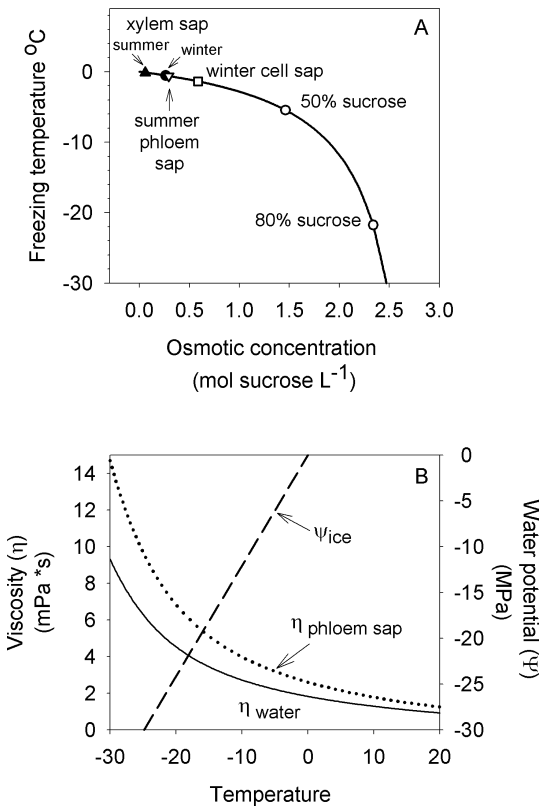

Figure 19.1 (A) The freezing point depression resulting from increased solute concentration, calculated as a 1.86˚C drop in freezing temperature per mol solute per kg water. Molal concentrations were converted to molar concentrations based on the molecular weight of sucrose. Approximate concentrations of summer (*filled triangle*) and winter (*filled circle*) xylem sap in sugar maple (Kozlowski and Pallardy, 1997), and summer phloem sap from castor bean, *Ricinus communis* (*open triangle*) (Taiz and Zeiger, 1998) are shown, as well as winter cell sap from cold-hardy ivy, *Hedera helix,* grown under 5˚C days and −1˚C nights (Hansen and Beck, 1988). Note that the freezing depression in the xylem is very small. Two sucrose solutions (% = g sucrose g^{-1} solution) are also shown that have osmotic concentrations equivalent to those that might be expected during winter in partially dehydrated living cells, including the phloem, in cold-adapted species as a result of extracellular freezing. (B) Relationship between viscosity and water potential of ice with temperature. Solid curve shows the exponential increase in viscosity with decreasing temperature for pure water (based on Lide, 1993); dotted curve is for a 10% sucrose solution (10 mg mL^{-1}) (based on Mathlouthi and Génotelle, 1995) of the same approximate osmotic concentration as measured phloem sap (Taiz and Zeiger, 1998). The linear decline in the water potential of ice is calculated as $\Psi_{ice} = RT/V_w^* \ln(P_{ice}/P_{liquid})$, where Ψ is water potential, R is the universal gas constant, T is temperature, V_w is the molar volume of water, and P_{ice} and P_{liquid} represent water vapor pressure of ice and supercooled water, respectively. This calculation agrees well with measured psychometric values (Hansen and Beck, 1988).

mic concentration and on interactions between the plasmalemma and cell walls (Guy, 2003).

Extracellular freezing protects cells from intracellular freezing but causes dehydration inside the cell proportional to the temperature below freezing (Kubler, 1983). For woody plants occurring in temperate regions, cellular dehydration is thought to be the primary cause of freezing injury (Tranquillini, 1982; Ashworth *et al.*, 1993; Webb and Steponkus, 1993; Fujikawa, 1997). The sensitivity to dehydration of the living cells in wood, particularly the xylem ray parenchyma cells that are more sensitive than other woody tissues, may ultimately determine freezing tolerance of woody species. Whereas cortical or cambial cells in most hardwood species respond to freezing in nature by extracellular freezing, cells of the xylem ray tissues tend to avoid the associated dehydration through supercooling (Fujikawa, 1997).

Supercooling

Deep supercooling in plant tissues prevents intracellular freezing while limiting the degree of cellular dehydration (Fujikawa *et al.*, 1996). Supercooling refers to the cooling of a liquid below the freezing temperature that is expected based on the solute concentration. It can occur readily in very small volumes of water, where surface properties influence the free energy of water, particularly in the absence of nucleation particles or agents that initiate ice-crystal formation. During supercooling, cells are not subjected to the effects of freezing, and cell functions can be maintained, albeit at a reduced rate. Supercooling is a common phenomenon in woody plants, both in leaves and the living cells of the xylem, including the xylem ray parenchyma cells (Guy, 2003). Limited supercooling to −10° C, referred to as shallow supercooling, has even been demonstrated experimentally in the xylem parenchyma of trees native to nonfrost tropical and subtropical zones, including *Ficus elastica, F. microcarpa, Mangifera indica, Hibiscus Rosa-sinensis,* and *Schefflera arboricola,* indicating that it may be an inherent property of the anatomical structure of wood (Kuroda *et al.*, 1997).

Shallow supercooling may occur without extracellular freezing but can quickly lead to rapid intracellular freezing, killing cells, if nucleation occurs. Deep supercooling generally occurs in combination with extracellular freezing and increasing solute concentrations inside cells. Both viscosity and surface properties of membranes and macromolecules influence the supercooling process. As temperatures decrease, the viscosity of liquid water increases exponentially (Fig. 19.1B) (Cho *et al.*, 1999). As solute concentration increases during extracellular freezing, water becomes even more viscous, particularly at low temperatures (Fig. 19.1B) (Mathlouthi and Génotelle, 1995). As a consequence, water molecules are slower to diffuse and to rotate so that ice nuclei are less likely to form. With high viscosity, attraction to hydrophilic surfaces also becomes increasingly important, par-

ticularly for water in small volumes, and water is less likely to diffuse from the region near a hydrophilic surface to a region where ice has already started to form. Increasing viscosity and hydrophilic membranes thus promote supercooling and decrease dehydration in cells (Wolfe *et al.*, 2002).

As subzero temperatures decline, the likelihood of ice nucleation increases (even in the absence of nucleating particles) until −40° C, when homogeneous nucleation of water occurs. This causes a breakdown of supercooling leading to intracellular freezing in the living cells although the −40° C limit can be lowered in proportion to the osmotic concentration of the cell sap or raised by the presence of heterogeneous nucleators (e.g., caused by bacteria or various injuries) (Guy, 2003). Some boreal hardwood species, including *Salix sachalinensis*, *Populus sieboldii*, *Betula platyphylla*, and *B. pubescens*, that grow in areas where the minimum air temperature reaches −50°C or below have xylem parenchyma cells that undergo deep supercooling in concert with extracellular freezing (Gusta *et al.*, 1983; Kuroda *et al.*, 2003). In these species, high osmotic concentration in the xylem ray parenchyma cells extends the limit of supercooling below −50°C, and these cells become only partially dehydrated. Other tree species that exist in extremely cold environments where the annual minimal temperature is significantly less than −40°C, including red osier dogwood (*Cornus sericea)* (Kuroda *et al.*, 2003), are generally thought to rely only on extracellular freezing, as this mechanism has the potential to allow survival even at the temperature of liquid nitrogen (−196°C) (Sakai and Larcher, 1987). Species that undergo extracellular freezing at such low temperatures must have mechanisms for dealing with the resulting severe dehydration stress.

Cold Acclimation

Sakai (1970) observed that under natural conditions, the freezing resistance of trees increases when the daily minimum temperature falls to subzero for a week and that the degree of freezing resistance depends on the air temperature at which the plants are wintering. In a series of experiments with different willow species, he showed that whether species actually tolerate freezing depends on the climate they experience and whether they have been cold acclimated. The acclimation process, or cold hardening, is often triggered in response to a decrease in the photoperiod (sensed by phytochrome) and exposure to cold temperatures. After growth ceases in response to declining photoperiod, some degree of freezing resistance often develops even without exposure to low temperature (Napp-Zinn, 1984). Subsequent changes occur in response to cold temperatures. What actually occurs during cold acclimation of plants? In woody species, this

process involves a suite of complex changes in anatomy and functioning of living and nonliving tissues, although not in areas of active growth. Growing shoots are cold sensitive and are not able to increase their freezing resistance even when acclimated to cold temperatures. In nongrowing tissues, the following changes have been observed in species that become cold acclimated:

- alterations in cell wall properties
- alterations in the plasma membrane, including lipid composition
- development of an amorphous layer of xylem parenchyma cells
- increased abscisic acid (ABA) concentration in different tissues and organs
- changes in gene expression and upregulation of dehydrins as well as enzymes involved in sugar metabolism
- accumulation of sugars in living and nonliving tissues

Cell Wall Structure

Composition and properties of cell walls change during cold acclimation, including increases in cell-wall thickness and tensile strength and decreases in pore size, changes in the amounts of lignin and suberin, and increases in cell-wall-associated proteins. These changes are important, as the walls are at the interface between extracellular ice and the cell protoplasm. Temperature-dependent changes in the freezing behavior of xylem parenchyma cells in hardwood species are thought to be controlled by changes in cell wall properties (Fujikawa and Kuroda, 2000; Fujikawa, 2002).

The porosity of the cell wall is thought to play a role in the flow of cellular water to extracellular ice. During cold acclimation in some woody species, deposition of pectin in the cell wall reduces the size of the microcapillaries in the walls, thus increasing dehydration resistance and preventing the growth of ice crystals into the cell. In the spring, pectin is enzymatically removed again, and supercooling to low temperatures is no longer possible (Wisniewski *et al.*, 1991). The rigidity of the cell wall prevents cell contraction and collapse during freezing that could otherwise occur owing to the growth of extracellular ice crystals and protects against membrane damage (Burke *et al.*, 1976; Rajashekar and Lafta, 1996). Rigid cell walls may also be important in preventing heterogeneous nucleation of ice formation, thus allowing deep supercooling (Wisniewski and Ashworth, 1985; Ashworth *et al.*, 1993). Phenolic cross-linking between cell wall polymers, deposition of lipids on the cell wall and deposition of extensin on the cell wall during cold acclimation may increase rigidity.

Plasma Membrane

The plasma membrane has been isolated as the primary site of freezing injury in many cold-sensitive plants (Stepkonkus *et al.*, 1983; Steponkus,

1984). The interaction between the plasma membrane and the cell wall differs between cold acclimated and non acclimated specimens of red osier dogwood, a highly cold tolerant woody species (Ristic and Ashworth, 1995). In subarctic trees, which tolerate temperatures below −40°C, low temperatures cause the synthesis of membrane lipids with less saturated fatty acids allowing membranes to remain flexible at low temperatures. Along with the cell wall, the plasma membrane is likely to be important as a barrier against the seeding of ice into the cells.

Amorphous Layer of Xylem Parenchyma

The xylem parenchyma cells that border vessels in angiosperms, called contact cells (see section on xylem refilling), are characterized by having a wall layer deposited between the plasma membrane of the parenchyma cell and the adjacent vessel-parenchyma pit membrane, called an amorphous layer or protective layer. This layer is likely to be important in the freezing resistance of plants as it may increase dehydration resistance. Seasonal alterations in the structure of this layer may affect its permeability and thus regulate the response of xylem parenchyma to freezing temperatures (Schaffer and Wisniewski, 1989).

Increased Abscisic Acid Levels and Altered Gene Expression

ABA is likely to play an important role in cold acclimation processes in many kinds of plants. ABA has a direct role in the response to cell desiccation that occurs during freezing, and it is also likely to be involved in the control of gene expression during cold acclimation. It has been hypothesized that low temperatures induce increased synthesis of ABA in plants, which in turn triggers the expression of genes involved in freezing tolerance. In a number of plants, including winter wheat, bromegrass, and arabidopsis, exogenous application of ABA induces cold-acclimation (e.g., Churchill *et al.*, 1998). In sugar maple, the ABA concentration of xylem sap was found to increase 10-fold in late autumn, reaching a maximum before maximum cold hardiness in buds and roots (Bertrand *et al.*, 1997). The rise in ABA could be induced by freezing dehydration (Hartung and Davies, 1991). The increased apoplastic ABA concentration could then influence gene expression during cold acclimation (Guy, 1990).

During the development of freezing tolerance in cold-hardened buds and roots of different species, upregulation of proteins in the dehydrin family have been observed after increases in ABA levels (Wisniewski *et al.*, 1996; Bertrand *et al.*, 1997; Sarnighausen *et al.*, 2002). Dehydrins are hydrophilic and heat stable and are thought to play a role in membrane stabilization and in the stabilization of the linkages between the cell wall and the plasma membrane during cold stress (Wisniewski *et al.*, 1999). Some of these proteins have been classified as antifreeze proteins (Wisniewski *et al.*, 1999),

which are known to aid in low temperature resistance of animals (e.g., Ewart *et al.*, 1999). In plants, they may prevent or restrict the growth of ice crystals and control the sites of ice formation (Griffith *et al.*, 1997), although they are unlikely to actually prevent plants from freezing, owing to their minimal impact on freezing depression (0.3°C) (Pearce, 2001).

Accumulation of Sugars

Proteins involved in starch-to-sugar conversion and in sucrose synthesis and degradation that control the sugar cycle in trees also appear to be upregulated during cold acclimation (Schrader and Sauter, 2002). Sugar accumulation in living and nonliving tissues during winter in cold-hardy trees has long been observed and is thought to be important in cold acclimation because it increases viscosity, which reduces ice crystal formation, helps stabilize membranes by binding to the free phosphate groups of membrane lipids, maintains respiration in living cells, and allows cell metabolism to recover after freezing (Schrader and Sauter, 2002; Guy, 2003; Wong *et al.*. 2003). It also lowers the freezing temperature of sap, although this has little effect until osmotic concentrations are quite high (Fig. 19.1A), as can occur in living cells during extracellular freezing. Upregulation of sucrose-phosphate synthase and sucrose synthase (which actually degrades sucrose), observed in *Populus,* may allow tight coupling of the regulation of sucrose biosynthesis and breakdown with temperature during cold acclimation in trees (Schrader and Sauter, 2002).

In sugar maple, starch is accumulated in the xylem ray tissues in late summer and early fall. As temperatures drop during the cold season, starch is hydrolyzed and soluble sugars accumulate. A sharp decline in the level of starch in the wood tissues corresponds to the accumulation of a large pool of sugars, including sucrose, fructose, glucose, stachyose, raffinose, and xylose. At the end of dormancy, the levels of soluble sugars decline and starch levels increase. Sugar concentrations increase again before leaf out, as carbon is mobilized for primary growth activities, including flowering and shoot and root growth (Wong *et al.*, 2003). This coincides with the timing of vessel repair and replacement (discussed later).

Impacts of Freezing on Water-Conducting Conduits of the Xylem

Xylem Tension and Conduit Size

In woody plants, freezing occurs in the apoplastic space of xylem conduits at much higher temperatures than inside the living cells of wood (Fujikawa *et al.*, 1996). In nonhardened plants, freezing exotherms of stems occur

between −1˚C and −5˚C, presumably indicating freezing of xylem sap. Because air is 1000 times less soluble in ice than in water, when sap freezes in xylem vessels and tracheids, air is released from solution and forms bubbles that become trapped in the ice (Scholander *et al.*, 1953). Upon thawing, the bubbles may dissolve back into the sap or expand causing conduits to embolize. Whether embolism occurs is thought to depend on the balance between the pressures exerted on the air-water meniscus.

A number of studies indicate that across diverse taxa, larger xylem conduits are more vulnerable to embolism by freezing than smaller conduits (Ewers, 1985; Sperry and Sullivan, 1992; Lo Gullo and Salleo, 1993; Davis *et al.*, 1999; Feild and Brodribb, 2001; Pittermann and Sperry, 2003). The main explanation is that larger bubbles form in larger xylem conduits. Larger bubbles are more difficult to dissolve upon thawing and dissolution time increases approximately with the square of the initial bubble diameter (Ewers, 1985; Yang and Tyree, 1992). The tension during thawing and the timing of the onset of tension upon thawing influences whether dissolution or expansion occurs (Tyree and Zimmermann, 2002). If the xylem pressure potential (P_x) is more negative than the surface tension forces acting to compress the bubble, then the bubble expands. Surface tension forces are given by Laplace's law, and are equal to $2^*T/r$, where T is the surface tension of water (0.0728 Pa m at 20˚C) and r is the bubble radius. If these forces overcome the tension in the xylem, the bubble contracts and dissolves. This can be seen from the following equation:

$$P_b = 2T/r + P_x \qquad (P_b >/= \text{pure vacuum})$$

(Yang and Tyree, 1992), where P_b is the internal pressure of the bubble. The larger the bubble and the lower the xylem pressure, the lower the bubble pressure, increasing the likelihood of expansion. In contrast, the higher the xylem pressure and the smaller the bubble, the more likely the bubble will dissolve. Assumptions and caveats of these theoretical expectations have been discussed in detail elsewhere (Sperry and Robson, 2001).

When data from similar diameter stems from various authors, representing a wide array of species, are plotted together, this broad relationship between vessel diameter and vulnerability to freezing is apparent, indicating a continuum of freezing sensitivity ranging from the southern hemisphere conifer, *Diselma archeri,* with a mean vessel diameter of 11.4 μm and no loss of conductivity after freezing, to the ring porous deciduous oak, *Quercus gambelii,* with a mean conduit diameter of 71 μm (hydraulically weighted diameter of 99 μm) and almost complete loss of conductivity after freezing (Fig. 19.2). The residual variation in the dependence of freezing-induced embolism on mean conduit diameter (Fig. 19.2) is high (either for a linear or a sigmoidal function), however, possibly as a result of differences in freezing and thawing conditions, which could change dynamics of

bubble formation. Alternatively, the loss of conductivity after freezing may be more directly related to the distribution of vessel diameters in a stem or to the hydraulically weighted vessel diameter (Fig. 19.2). If most of the stem conductance is through a small number of large diameter vessels that have a high probability of embolizing after a freeze-thaw event (ring porous species), these species show higher losses of conductivity after freezing even if their average vessel diameter is relatively low, as flow rate in a conduit is estimated to be proportional to the 4th power of the radius according to the Hagen-Poiseuille relationship. The correlation between conduit diameter and loss of conductivity also depends on having a large range of mean conduit diameters. Among 17 species of co-occurring Florida oaks, which share important similarities in wood and anatomical properties and for which diameter variation falls within a relatively restricted range, loss of conductivity after freezing is not dependent on vessel diameter (Cavender-Bares and Holbrook, 2001). Nevertheless, these values fall within the expected range of the larger sample of woody species (Fig. 19.2).

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Other aspects of xylem anatomy may also be important. Conduit lengths may be important depending on the physics of bubble formation (i.e., whether many bubbles form throughout the conduit that can coalesce

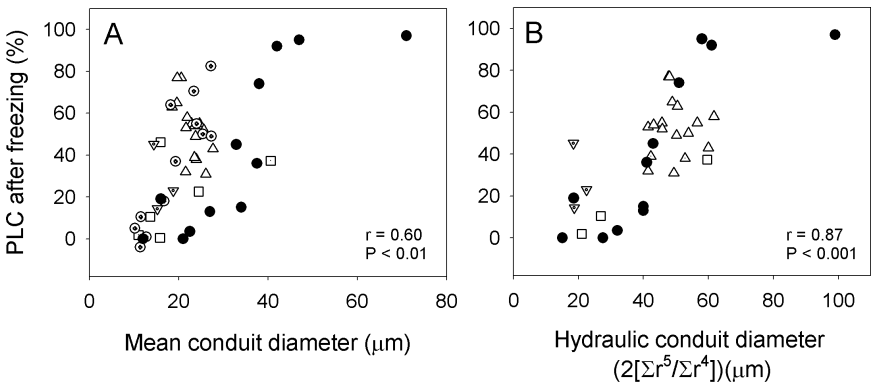

Figure 19.2 Relationship of percent loss of hydraulic conductivity in woody plant stems to mean conduit diameters from multiple authors (A) and hydraulically weighted conduit diameter, calculated as 2^* ($\Sigma r^5/\Sigma r^4$), where r is conduit radius, for available data from the same authors (B). Freezing temperature, xylem tension and freeze/thaw rates are indicated with authors for each symbol. Linear correlation coefficients are given in each plot. A sigmoidal function would be preferred over a linear fit if there is a conduit diameter corresponding to a cavitation threshold, although non-linear functions fit poorly to the combined data.

{au: fig 2 doesn't show A B distinctions; pls clarify}

to form large bubbles or whether they form in longitudinal files that do not coalesce) (Pittermann and Sperry, 2003). In Norway spruce, Mayr *et al.* (2003b) found that leader shoots have significantly lower freezing-induced embolism, despite larger diameter tracheids, than twigs. They attributed the lack of freezing embolism in leader shoots to smaller pit dimensions and lack of compression wood.

Temperature Dependence of Xylem Cavitation

In addition to conduit diameter, xylem sensitivity to freezing appears to be dependent on the minimum temperature experienced (Lo Gullo and Salleo, 1993; Kuroda *et al.*, 1997; Pockman and Sperry, 1997; Pittermann and Sperry, 2003) (Ball, personal communication; J. Cavender-Bares, P. Cortes, R. Joffre, S. Rambal, unpublished), as well as on the rate of freezing (Kikuta and Richter, 2003) and thawing (Sperry, 1995; Cordero and Nilsen, 2002) (see Chapter 20), and the number of freeze-thaw cycles the {au: ok?} plant has experienced previously (Lemoine *et al.*, 1999; Mayr *et al.*, 2003a). Various authors have shown that loss of conductivity to freezing can vary threefold with a temperature decline from −5° C to −15° C, depending on the species (Fig. 19.3). The redistribution of water as extracellular freezing occurs into other parts of the wood may increase tension in the xylem that would increase the likelihood of bubble expansion on thawing. Kikuta and Richter (2003) showed that ultrasonic acoustic emissions (typically thought

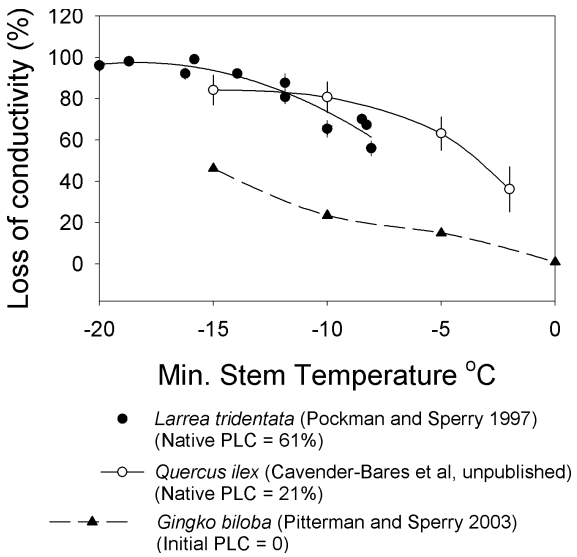

Figure 19.3 PLC in different species with decreasing minimum freezing temperature.

to correlate with cavitation events) become much more frequent in rachides of non–cold-hardened black walnut below −12° C, well after the prominent xylem exotherm occurred, signaling potentially important changes in xylem that could contribute to reduced function at lower temperatures (Fig. 19.4). One interpretation of these results is that numerous freezing events, not resulting in measurable exotherms, occurred in very small diameter vessels or fibers as a result of the breakdown of supercooling with decreasing temperatures. Alternatively, these signals could have resulted from disruption of living cells of the xylem, such as the xylem ray parenchyma. The number of acoustic emission was much lower in saturated stems than in unsaturated stems (Ψ = ~ −0.7 MPa), indicating that tension in the xylem increased the number of acoustic emissions.

LoGullo and Salleo (1993) showed that within a given stem, larger vessels were more likely to embolize than smaller vessels as a result of freezing, and the number of embolized vessels increased with decreasing temperature. They identified critical vessel diameters for a given temperature that were susceptible to xylem embolism. They argued that narrow conduits are likely to stay functional at colder temperatures than larger vessels because narrow vessels contain liquid water at lower free energy, and therefore have a lower freezing point and hence a greater capacity for supercooling. LoGullo and Salleo (1993) also found higher recovery of xylem embolism after freezing in smaller vessels, which they speculated to be the result of redissolution of air in sap after high root water absorption relative to transpiration. The per-

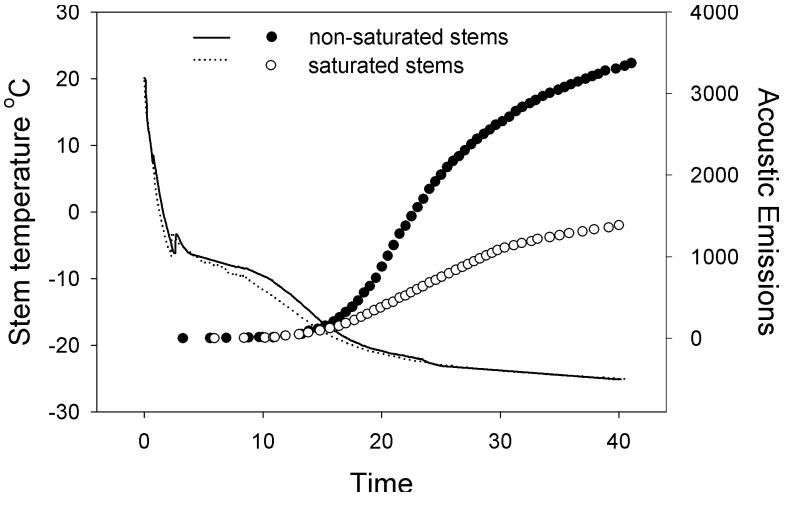

Figure 19.4 Rise in ultrasonic acoustic emissions with decreasing stem temperatures below the freezing point in stems of *Juglans nigra* for both a saturated and an unsaturated stem. (Replotted from Kikuta and Richter, 2003.)

{figs 3 and 4 appear to be reversed; pls clarify here and in text}

centage of xylem conduits that recovered was negatively correlated with conduit diameter, presumably because it is more difficult to expel larger bubbles from wider xylem conduits (Lo Gullo and Salleo, 1993).

The interaction of stem diameter (surface area-to-volume ratio), vessel diameter and volume, xylem tension, freezing temperature, and anatomical features that influence the way water is redistributed in the wood during freezing are likely to influence the degree of susceptibility of xylem to freezing under natural conditions. In *Fagus sylvatica*, apical shoots freeze more quickly than older shoots with larger vessels (Lemoine *et al.*, 1999), most likely because of higher surface-to-volume ratios of apical versus second- or third-year shoots. Apical shoots have narrower vessels than those of older shoots, but show higher embolism after freeze-thaw events. Lemoine *et al.* (1999) observed that water exudation as a consequence of volume expansion and redistribution of water during freezing lowered the water content of the smaller shoots. They hypothesized that this induced higher xylem tension on thawing and triggered embolism formation in the apex. Although bubbles were likely to be smaller in these terminal shoots, the capillary pressure they developed was not high enough to compensate for the decrease in xylem pressure. They concluded that embolism formation depends more on dynamics of sap freezing than on xylem characteristics.

Links Between Xylem Properties, Phenology, and Climate

Xylem vulnerability to freezing stress has been shown to vary considerably among species, and xylem properties appear to be correlated with phenology (Lechowicz, 1984; Wang *et al.*, 1992; Cavender-Bares and Holbrook, 2001a) and climatic distribution (Noshiro and Baas, 2000; Cordero and Nilsen, 2002). Hydraulic properties and patterns of vulnerability to freezing show biogeographical patterns, suggesting that freezing-induced embolism is likely to be an important factor limiting species ranges. Xylem conduit length and diameter are significantly negatively correlated to latitude among species within the genus *Cornus* with longer vessels (and fibers) and larger-diameter vessels occurring in warmer climates (Noshiro and Baas, 2000). Among congeners of *Rhododendron*, species from habitats with a higher frequency of freeze-thaw events have smaller vessel sizes (Cordero and Nilsen, 2002). However, the *Rhododendron* species with the smallest mean (or hydraulically weighted) vessel diameter, *R. catawbiense*, is the most sensitive to freezing. Refilling following freeze-thaw events is apparently the main mechanism for overcoming freezing stress for this plant and is more important than preventing embolism (Cordero and Nilsen, 2002).

Mechanisms of Maintaining or Recovering Xylem Function

To survive freezing, plants must minimize embolism to allow continued function or resume xylem function following embolism. Conifers tend to

minimize xylem dysfunction by having very narrow conduits. After a freeze-thaw event, only a few tracheids with big air bubbles cavitate (Sucoff, 1969). As these bubbles expand, tension is released, which allows bubbles in the surrounding tracheids to dissolve. In distal shoots, embolism may protect the older parts of the branch from freezing damage. Still, to maintain winter activity in very cold climates, some transport must continue even while xylem sap is frozen. Continued water transport in frozen wood may be possible through cell wall capillaries at temperatures below freezing (Sparks *et al.*, 2000). Also, the process of freezing and thawing of stems can physically move water within the xylem (Zimmermann, 1983). During freezing, the increase in volume forces water to move centripetally, either from smaller to larger stems or into the heartwood (Robson and JA, 1993). In several northern species, including lodgepole pine and western larch, more than 25% of the water in wood was liquid even at −15˚C. This unfrozen water is mainly found within the cell wall and may be available for transport (Sparks *et al.*, 2000).

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Deciduous broadleaved trees, which are dormant in winter, tend to be more vulnerable to winter cavitation and may contain less liquid water at temperatures below freezing than wood of conifers. Rather than maintaining xylem function during winter, they recover it after the cold season either by the replacement of embolized vessels with new functional vessels or by refilling of embolized vessels. All plants with secondary cambium can replace xylem, but its efficiency during bud break depends on the timing of radial growth resumption (Ameglio, 2002). Some species, but not all, use the strategy of refilling embolized vessels through positive pressures in the xylem. For pressures to develop in the xylem vessels, an osmotic pressure difference must develop between the vessels and the neighboring compartment, the symplast (contact cells and xylem parenchyma), separated by a semipermeable cell membrane (Yang and Tyree, 1992).

Walnut and maple exhibit positive pressures in the xylem sap during winter. These are associated with high xylem sap sugar concentration (Ameglio and Cruiziat, 1992; Ameglio *et al.*, 2001b). Maple is unusual in that its xylem takes up water upon freezing, and xylem tension is reduced or even positive upon thawing, which reduces embolism formation (Sperry *et al.*, 1988; Tyree, 1983). In temperate, ring-porous oaks and in peach, however, positive xylem pressures have never been observed (Ameglio *et al.*, 2002). Freezing injury to roots that prevents development of positive pressure in the xylem after spring thawing can impair the ability of some species to refill vessels and recover from freezing-induced xylem embolism (Zhu *et al.*, 2002).

In many species, including most hardwoods, sugar concentration in the xylem sap is regulated by the xylem axial and ray parenchyma, which are living cells with large pits connecting them to vessels. These cells are the sites of sugar secretion in the xylem sap in spring and are involved in car-

bohydrate metabolism and the translocation, storage, and mobilization of nutrients (Schaffer and Wisniewski, 1989). Hence, xylem parenchyma are likely to govern the ability of trees to refill embolized xylem vessels after freeze-thaw events. Temperature may determine whether xylem parenchyma secrete or absorb sugars, with secretion dominating at low temperatures. At low, nonfreezing temperatures, the starch that is stored in parenchyma cells in the xylem is converted to sugars, particularly sucrose (Marvin *et al.*, 1967) and is then secreted by contact cells into the xylem sap (Ameglio and Cruiziat, 1992). In many temperate trees, positive xylem pressures are associated with high sugar concentrations in the xylem sap in the early spring, but not in the winter (Essiamah, 1980). Such trees have a period in the early spring when starch is hydrolyzed to sugars and released in the xylem sap.

Defoliation of walnut trees reduced winter osmotic concentrations and xylem pressures (Ameglio *et al.*, 2001b). The role of osmotic concentration in reversing winter embolism may explain why defoliated trees have a reduced ability to survive winter. Any factor, such as insect attack, pathogens, or severe summer drought, that results in a reduction of stored carbohydrates can reduce winter xylem pressures, which would reduce the ability of the plant to reverse embolism (Ameglio *et al.*, 2001b).

Ameglio *et al.* (2001a) found that the osmotic concentration of the xylem sap doubled in response to a freeze thaw event and the symplastic stem water content decreased as a result of sucrose secretion by contact cells. The increase in osmotic concentration was followed by xylem pressure increases as xylem sap drew water from the living cells of the xylem, and possibly from other unfrozen parts of the stem and roots. Surprisingly, these pressures increased almost sixfold over the value predicted by enhanced osmotic concentration alone (Ameglio *et al.*, 2001a). They hypothesized that some of the unexplained additional pressure might also have been associated with the expansion of water during the phase change from liquid to solid. The ice within vessels could have exerted pressure on the surrounding fluid until the ice was completely melted. Alternatively, changes in gas pressures with temperature in the xylem fibers could have caused water from the fibers to be expulsed.

Impacts of Cold Temperatures and Freezing on the Phloem

Physical Effects of Low Temperatures

Transport in the living phloem of woody plants is driven by mass flow from high pressure to low pressure regions throughout the phloem network (Muench, 1930; van Bel, 2003). As negative pressures do not occur in the

phloem, freezing-induced embolism is not a risk as it is in the xylem. Yet low temperatures pose other problems for phloem transport. Phloem sap is significantly more viscous than xylem sap, and phloem viscosity can increase by an order of magnitude with decreasing temperatures (Fig. 19.1A), posing significant limitations to bulk flow. Intercellular diffusion rates also decrease with colder temperatures, and functioning of organelles in companion cells that regulate phloem transport may be impaired. Plasmodesmata are thought to narrow in response to cold, reducing the efficiency of symplastic transport (Gamelei *et al.*, 1994). Decreasing sink strength with reduced respiration and growth activity in winter should also reduce the pressure gradient along the phloem pathway and limit phloem transport (Hoffmann-Thoma *et al.*, 2001). Consequently, in deciduous plants, as growth ceases in winter, the phloem is likely to become inactive. In temperate woody dicots, new phloem (inner bark) is put down by the vascular cambium in the spring and previous phloem collapses as xylem expands. Phloem differentiation begins in the buds and new shoots and extends basipetally downward. It can precede or follow xylem development by several weeks in the spring depending on the species. Annual phloem growth rings may often be observed in the bark of temperate trees. Renewal of phloem tissue and collapse of older sieve tubes is thought to occur in a similar way in tropical evergreen species (Zimmermann, 1961). In most conifers, phloem activity and differentiation continue through the winter (Priestley, 1930). An interesting question is the extent to which previous year phloem can be reused in different species. In long-lived monocots, phloem sieve tubes can survive multiple decades owing to the maintenance and support of the companion cells (van Bel, 2003).

Extracellular Freezing and Bark Shrinkage

At freezing temperatures, bark shrinks as a result of water movement out of sieve tube elements into the apoplast (Zweifel and Hasler, 2000; Ameglio *et al.*, 2001a). Cortical cells of phloem tissue, in contrast to xylem parenchyma, contract in response to freezing as extracellular ice forms and cells become dehydrated with the outflow of cellular water (Ashworth *et al.*, 1993). The lignified xylem parenchyma cells have thicker cell walls than that of bark tissue and are also attached to adjacent cells in all directions, which prevents contraction. In Norway spruce, a subalpine conifer, the loss of water in the bark (including in the cambium, phloem, and parenchyma) is dependent on contact with the xylem (Zweifel and Hasler, 2000). Exposed twigs in the periphery of the tree are likely to freeze first when the temperature drops below the freezing point. Once freezing begins, nucleation (seeding) occurs and freezing spreads to other parts of the tree. Partial freezing in the xylem can cause a strong water potential gradient between the phloem and the xylem, causing water to move into the xylem

from the phloem and allowing supercooling to occur in the phloem, protecting the live phloem cells from freezing damage. Increased osmotic concentration in the phloem allows for rapid rehydration after thawing. Zweifel and Hasler (2000) hypothesized that Norway spruce can remain active in winter because on sunny days, the crown gathers enough warmth to rehydrate the bark in the upper tree, even as the lower stem remains frozen. In the reactivated part of the crown, small amounts of photosynthesis and transpiration can occur and water is withdrawn from internal reserves, rather than from the soil. As the winter progresses, the crown and the bark of the upper stem become more dehydrated than the stem parts near the ground.

In contrast to Norway spruce, bark contraction in black walnut is not dependent on contact with the xylem (Ameglio *et al.*, 2001a). Extracellular water in the bark freezes first since it has a lower solute concentration than intracellular vacuolar and cytoplasmic water. The extracellular ice crystals then draw water out of the living bark cells through the plasma membrane. The porous bark allows the ice to expand even as the water loss in the living cells causes bark contraction. Contraction increases as plants become cold hardened, presumably because extracellular freezing increases relative to intracellular freezing.

Symplastic versus Apoplastic Phloem Loaders and Climatic Distribution

The hypothesis that cold stress may have been important in driving the evolution of an apoplastic loading strategy arose from the observation that plant species with high plasmodesmatal frequency (thought to be correlated with symplastic loading) evolved in tropical climates while plants with minor vein anatomy consistent with apoplastic loading evolved more recently in temperate climates (Gamelei, 1989). The corollary to this hypothesis is that apoplastic loaders may have greater transport efficiency at low temperatures (Gamelei *et al.*, 1994) and that symplastic loaders should be more vulnerable to cold temperatures than apoplastic loaders, which would be evidenced by reduced export efficiency from leaves and starch buildup in winter chloroplasts of mesophyll and bundle sheath cells. One of the underlying assumptions is that the greater the number of plasmodesmata at a given cellular interface, the greater is the potential for symplastic transport through that interface. However, species that are classified as symplastic loaders on account of plasmodesmatal connectivity may turn out to load differently (see Chapter 3 in this volume; Turgeon and Medville, 1998).

There are several reasons why symplastic species and species with greater numbers of plasmodesmata may be more vulnerable to cold stress. There is some evidence that translocation through plasmodesmata begins to be inhibited at 10° C and blockage occurs, with the temperature minimum of

symplastic transport thought to be between 2 and 4° C (Geiger and Sovonick, 1975). It has also been suggested that apoplastic loading, which requires a proton pump, could be more active at lower temperatures than symplastic loading, which depends on diffusion (Gamelei, 1991). Many symplastic loaders transport raffinose and stachyose and other raffinose family oligosaccharides (RFO) in addition to sucrose. These are synthesized from sucrose and galactose in intermediary cells and form a polymer trap that allows sucrose to diffuse into intermediary cells and into sieve elements while preventing large RFOs to diffuse back into the mesophyll. Raffinose shows much lower solubility at low temperatures than sucrose and should be poorly transported at cold temperatures (Turgeon, 1995), although other RFOs apparently have higher solubility at low temperatures (Lambers *et al.*, 1998).

However, recent experiments show no difference in cold sensitivity between symplastic and apoplastic loaders (Hoffmann-Thoma *et al.*, 2001). In a study of three broadleaved evergreen species that were shown to be symplastic loaders, reduced phloem loading was not observed in winter, and sugar export was maintained in cold-acclimated leaves. Phloem loading type was based on transport sugars and limitation by p-chloromercuribenzenesulfonic acid , which impairs sucrose carriers involved in apoplastic loading. During periods of cold temperatures, sugar exudation actually increased (Fig. 19.5). In addition, no damage to intermediary cell vacuoles or other structures were found after exposure to natural freezing episodes (–9 to –11° C). Hoffmann-Thoma *et al.* (2001) hypothesized that the additional sugar export in winter was necessary for the energy requirements of cold acclimation, including the production of solutes, osmotic adjustment, protein synthesis, and reorganization of membrane lipids. Starch did not accumulate in winter chloroplasts of mesophyll and bundle sheath cells, and much higher amounts were found in these cells in summer.

Low temperatures may impose greater limits on the symplastic pathway, but cold hardening is able to overcome these limitations. In plants from eight families, including four apoplastic loaders and four symplastic loaders, no significant differences were observed in response to chilling at 10° C, and there was no evidence that phloem loading was impaired in symplastic species (Schrier *et al.*, 2000). Phloem loading was fully operative at 10° C, and there was no difference in exudation of sugars between symplastic and apoplastic species. For all species, however, export of sugars in response to long-term exposure to 10° C was slower, as carbohydrates were lost more slowly and phloem loading was slightly lower. Starch content in leaves increased after long-term exposure to low temperatures in all plants. In all 10° C cold-shocked plants, cold sensitivity was apparent as indicated by a reduced export of sugars and total nonstructural carbohydrate. This sensitivity was explained by a possible cold-induced narrowing of the plasmodesmata in the trajectory mesophyll to sieve element (Schrier *et al.*, 2000). Thus,

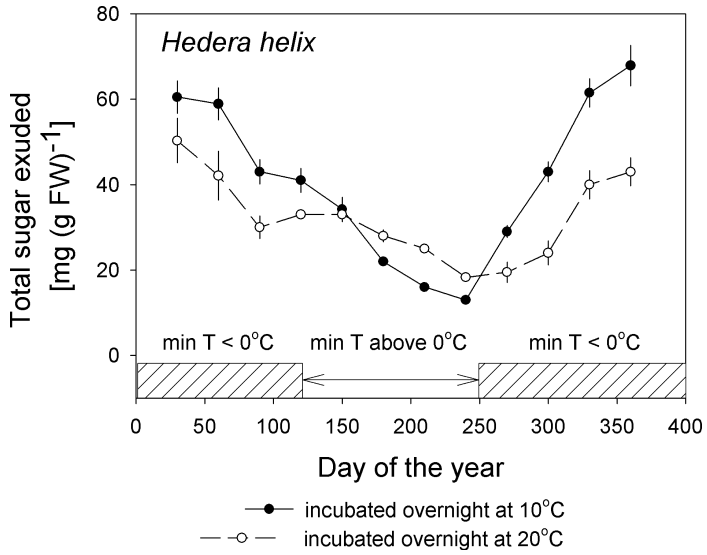

Figure 19.5 Sugar exudation levels of leaves in different seasons for the evergreen symplastic phloem loading species, *Hedera helix*, growing outdoors but incubated overnight at either 10˚ or 20˚C. For each experiment, total sugar quantity of overnight exudates was analyzed by high-performance anion exchange for at least four leaves of each species for each chamber temperature. Bar at bottom indicates timing of when daily minimum temperatures were below zero. Redrawn from Hoffman-Thoma *et al.* (2001).

low temperatures clearly impact phloem transport, but symplastic phloem loading is not more vulnerable to chilling stress than apoplastic loading.

Conclusions

Freezing and cold temperatures pose clear problems to living tissues and long distance transport systems in plants. These physical limitations are partially overcome by cold acclimation in cold-hardy plants, which involves a suite of biochemical and physiological transformations triggered by photoperiod and cold temperatures. The impacts of freezing on xylem transport depend in large part on xylem vessel diameters. Yet other anatomical features of xylem that influence their vulnerability to freezing embolism are only beginning to be understood. The mechanisms by which declining temperatures below freezing continue to impact xylem function are not well understood. Much is left to be learned about the influence of the redistribution of water during freezing on water content and tension in

xylem, and how this affects dynamics of cavitation and sap flow. For example, how does freezing tolerance of living xylem parenchyma influence xylem function? Although the impacts of freezing on living cells and on xylem in woody plants have been intensively studied, much less is known about the effects of subzero temperatures on phloem and its role, if any, in regulating sugar mobilization and xylem repair in the spring. How active is the phloem in winter across different plant taxonomic groups, and what role does the phloem play across diverse taxa in cold acclimation including in the transport of ABA and synthesized proteins? Studies that can address the impacts of freezing on cellular processes and on long-distance transport in both the xylem and the phloem will help provide an integrated understanding of plant function in response to freezing stress.

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