Freezing Stress Response in Woody Tissues Observed Using Low-Temperature Scanning Electron Microscopy and Freeze Substitution Techniques[1]

Stephen R. Malone and Edward N. Ashworth*

Center for Plant Environmental Stress Physiology, Department of Horticulture, Purdue University, West Lafayette, Indiana 47907

ABSTRACT

The objective of the current research was to examine the response of woody plant tissues to freezing stress by using scanning electron microscopy (SEM). Nonsupercooling species red osier dogwood (*Cornus stolonifera* Michx.), weeping willow (*Salix babylonica* L.), and corkscrew willow (*Salix matsudana* Koidz. f. *tortuosa* Rehd.) survived freezing stress as low as −60°C. Cell collapse of ray parenchyma cells of these species was expected but did not occur. It was concluded that ray parenchyma cells of these species do not fit into either the supercooling or extracellular freezing classifications. Tissues from flowering dogwood (*Cornus florida* L.), apple (*Malus domestica* Borkh. cv "Starking III"), red oak (*Quercus rubra* L.), scarlet oak (*Quercus coccinea* Muench.), and red ash (*Fraxinus pennsylvanica* Marsh) were confirmed as supercooling species, and did not survive exposures below −40°C. Ray parenchyma cells of these species did not collapse in response to freezing stress, as was expected. Cell collapse along the margins of voids were observed in bark of all seven species. Voids were the result of extracellular ice crystals formed in the bark during exposure to freezing stress. Tissues prepared by freeze substitution techniques were found to be adequately preserved when compared to those prepared by conventional fixation and low temperature SEM techniques. A freezing protocol for imposing freezing stress at temperatures lower than experienced naturally in the area where the study was conducted was developed that produced responses comparable to those observed in specimens collected in the field during natural freezing events.

Woody plant tissues that survive freezing temperatures have been classified into two distinct groups based on their freezing behavior: those that exhibit the deep supercooling characteristic and those that exhibit extracellular freezing (7–9, 11). These classifications were based primarily on results obtained using calorimetric and nuclear magnetic resonance methods and relating the results to the temperature range over which injury was observed (3, 4, 6–11, 14, 20–22).

Deep supercooling species were moderately hardy, and did not survive below −40°C. When analyzed by DTA,[2] two exotherms were detected. An HTE, which occurred a few degrees below 0°C, was thought to be due to freezing in vascular tissue and was not correlated with injury (7, 8, 22). An LTE was detected near the homogeneous nucleation temperature (−40°C). This exotherm was correlated with death of xylem ray parenchyma cells (3, 7–10, 16, 21, 22). It was proposed that water within these cells supercooled to temperatures approaching the homogeneous ice nucleation temperature before freezing. Cell death presumably resulted from intracellular ice formation and cells would be expected to maintain their original shape and volume during a freezing stress (3, 7–9).

Species that do not exhibit the supercooling characteristic ranged in hardiness from a few degrees below 0 to −196°C (23). It was thought that ice formation was initiated in extracellular spaces. As tissues were further cooled, a water potential gradient was established that caused water to be removed from the cells to the growing extracellular ice crystal. This progressive dehydration resulted in a concentration of the intracellular solution and a reduction in cell volume. Reduction in cell volume would be expected to appear as cells with collapsed cell walls. Both extracellular ice crystals and cell collapse should be visible in tissues that freeze extracellularly. Direct observations of woody plant cell response to freezing are limited. That cells collapse and undergo extracellular freezing has been inferred from studies conducted using red blood cells and herbaceous plant material (17–19, 24). Cell collapse and extracellular freezing have been observed in bark tissue of apple (2), but not in xylem ray parenchyma cells.

Both freezing responses have been observed concurrently in different tissues of the same species. In apple (*Malus domestica* Borkh. cv "Golden Delicious"), xylem ray parenchyma and pith cells did not collapse in response to freezing, and cell death was attributed to the intracellular freezing of supercooled water (2, 15, 16, 21, 22). In contrast, injury to bark tissue was not correlated with a distinct freezing event (21, 22). Bark cells were observed to have collapsed in response to a freezing stress. This was consistent with the idea that the bark tissue froze extracellularly. Both the presence of

[2] Abbreviations: DTA, differential thermal analysis; HTE, high temperature exotherm; LN₂, liquid nitrogen; LTE, low temperature exotherm; LTSEM, low temperature scanning electron microscopy; SEM, scanning electron microscopy; TZ, tetrazolium chloride.

extracellular ice and cell collapse were confirmed by using LTSEM (2).

The objective of the present study was to test the hypothesis that cells in tissues that do not exhibit an LTE undergo extracellular freezing and cell collapse in response to a freezing stress. Red osier dogwood and weeping willow have been previously classified as extracellular freezing species (11, 12). We confirmed this classification using DTA. Based on DTA experiments done in our laboratory, corkscrew willow was also included as an extracellular freezing species. In these species, bark and xylem ray parenchyma cells would be expected to appear collapsed in specimens subjected to a freezing stress.

Flowering dogwood, apple, and red oak exhibit the deep supercooling characteristic. Therefore, xylem ray parenchyma cells would not be expected to collapse in response to freezing stress. Bark cells should freeze extracellularly as previously reported in apple (2). Scarlet oak and red ash exhibit the deep supercooling characteristic but can further harden with prolonged subzero exposure (12). An intermediate response of xylem ray parenchyma cells would be anticipated. Bark tissues of all species should freeze extracellularly and exhibit cell collapse.

MATERIALS AND METHODS

Plant Material

Plant material included: red osier dogwood (*Cornus stolonifera* Michx.), weeping willow (*Salix babylonica* L.), corkscrew willow (*S. matsudana* Koidz. f. *tortuosa* Rehd.), flowering dogwood (*C. florida* L.), apple (*Malus domestica* Borkh. cv "Starking III"), red oak (*Quercus rubra* L.), scarlet oak (*Q. coccinea* Muench.), and red ash (*Fraxinus pennsylvanica* Marsh).

Twigs of the previous summer's growth were collected from plantings adjacent to the Purdue University campus. All plants appeared to be healthy. Collections were made on several dates beginning in late fall, shortly before the first freeze, and continuing through the winter until plants began to deacclimate in the spring.

Differential Thermal Analysis

Stem tissues were removed on the day of each experiment, immediately wrapped in moistened paper towels, and enclosed in aluminum foil envelopes. Specimens were packed in snow or crushed ice and transported to the laboratory. DTA technique was a modification of that used by Quamme *et al.* (22). Segments of stem tissues, approximately 5 mm in length, were cut on moistened filter paper. After the bark was removed, the segments were placed in contact with the junction of a 40-gauge Cu-constantan thermocouple by means of a small aluminum foil cup. This assembly was then placed in a test tube inserted in an aluminum block in a −80°C freezer (Puffer Hubbard, New York, NY). Five samples of the same species were run simultaneously. The block temperature was controlled by a programmable temperature controller (Omega Engineering, Inc., Stamford, CT, model CN-2010). The block was cooled at 20°C/h from 5 to −60°C. Sample temperatures were recorded using a multichannel strip-chart recorder (Lin-

seis, Inc., Princeton, NJ, model L2065-6). The temperature of freezing events was determined by comparing the differential output of individual samples with the temperature of an oven-dry reference sample.

Tissue Survival Tests

A battery of hardiness tests were performed on control and frozen specimens to determine the temperature range over which cells were killed and to confirm if tissues were alive before the stress treatment was applied. Freezing stresses were applied to specimens at either 0, −20, −30, −40, −50, −60°C, or plunged directly into LN₂. Twigs were wrapped in moistened Kimwipes, placed in aluminum foil envelopes, and loaded into Thermos bottles in a walk-in freezer and cooled to approximately −15°C. Specimens were subsequently transferred to the −80°C freezer for further cooling. Sample temperatures were monitored and specimens were removed at appropriate test temperatures and thawed overnight in a refrigerator. Cooling rate was approximately 10°C/h. Viability of bark and wood were assessed separately. A visual rating scale was used for the tetrazolium and tissue browning tests where "0" indicated no living cells and "4" indicated no dead cells.

In the TZ test, tissues were considered alive if a red color was evident in individual cells following overnight incubation at room temperature in a 0.6% (w/v) TZ solution buffered to pH 7.4 (25). Tissue browning test results were visually evaluated after allowing tissues to incubate at room temperature and high humidity for 2 d.

Collection of Naturally Frozen Twigs

Specimens were collected outdoors while subjected to freezing temperatures of approximately −5 and −10°C during January, February, March, and December 1989. Plants had been exposed to freezing temperatures for at least 24 h prior to collection. Air temperatures were measured using a portable temperature monitor with a thermocouple placed as near the twigs to be collected as possible. To minimize thawing during handling, tools and working surfaces were equilibrated to ambient outdoor temperatures prior to touching specimens. Specimens for freeze substitution or LTSEM experiments were cut on a cardboard working surface and immediately quench frozen in melted Freon 12 (−150°C). Specimens were transferred to LN₂ and subsequently transported to the laboratory or SEM facility.

Laboratory Freezing Protocol

It was necessary to develop a method of duplicating natural freezing response under conditions where the rate of freezing could be controlled and specimens could be cooled to lower temperatures than normally experienced in our area during the winter. Specimens to be processed by freeze substitution were cut into 1 mm thick cross-sections on moistened filter paper. Samples for LTSEM were prepared by cutting cross-sections into thirds and loading these smaller pieces into 1 or 1.5 mm (i.d.) × 3 mm long Ag tubes. Specimens were placed on a metal plate floating in a programmable temperature bath

set at 1°C (Neslab Instruments, Inc., Newington, NH, model RTE-210). The plate was lightly coated with vegetable oil to keep specimens from sticking to the plate and to help reduce desiccation. To avoid surface drying, inverted Petri dishes were placed over the specimens. Ice chips were placed near the specimens to promote nucleation. Plate surface temperatures were constantly monitored throughout a run using an attached thermocouple. Specimens were allowed to equilibrate at 1°C for 30 min, then cooled at 5°C/h, and harvested at the appropriate test temperature with precooled tweezers. This method was useful for freezing to approximately −20°C.

To freeze tissues to colder temperatures, an aluminum block with a depressed flat surface was placed in the −80°C freezer. Block temperature was controlled with the same controller and heater used for DTA. Specimens were prepared and placed on the freezing surface as described above. A fitted aluminum lid was placed over the Petri dish to maintain uniform temperature. Following equilibration at 1°C, specimens were cooled from 1 to −20°C at 5°C/h. Samples were allowed to equilibrate at −20°C for 1 to 2 h before being collected. The remaining specimens were subsequently further cooled at 5°C/h to −60°C, and held at least 1 h until harvested.

Conventional Fixation

Control specimens were collected from the field as described previously and chemically fixed. Cross-sections were cut on moistened filter paper and placed in 3.5% glutaraldehyde in 25 mM sodium phosphate buffer (pH 6.8). After 3 h, the tissues were rinsed in three changes of buffer, and postfixed in 1% OsO_4 for 1 h (5). Tissues were then dehydrated in a graded ethanol series and held in anhydrous ethanol pending further processing.

Specimens were removed from the ethanol, blotted, then placed onto a metal plate submerged in LN_2. To reveal a

Table I. *Evaluation of Hardiness to Freezing Stress by TZ and Tissue Browning Tests of Nonsupercooling Species Red Osier Dogwood and Weeping Willow Collected in February 1990*

Species	Treatment	TZ		Browning	
		Wood	Bark	Wood	Bark
	°C				
Red osier	0	4[a]	4	4	4
dogwood	−20	4	4	4	4
	−30	3	3	4	4
	−40	3	3	4	4
	−50	3	3	4	4
	−60	3	3	4	4
	LN_2	0	0	0	0
Weeping willow	0	3	4	4	4
	−20	4	4	4	4
	−30	4	4	3	3
	−40	3	3	3	3
	−50	3	3	1	1
	−60	3	3	1	1
	LN_2	0	0	0	0

[a] Rating scale: 0 = no cells appearing alive, 4 = all cells appear alive.

Table II. *Evaluation of Hardiness to Freezing Stress by TZ and Tissue Browning Tests of Supercooling Species Flowering Dogwood and Red Oak Collected in February 1990*

Species	Treatment	TZ		Browning	
		Wood	Bark	Wood	Bark
	°C				
Flowering	0	4[a]	4	4	4
dogwood	−20	3	3	4	4
	−30	2	2	3	4
	−40	2	2	3	3
	−50	1	1	2	3
	−60	1	1	2	3
	LN_2	0	0	1	1
Red oak	0	4	4	4	4
	−20	4	3	4	4
	−30	4	3	2	4
	−40	2	3	2	4
	−50	2	3	2	3
	−60	0	2	1	2
	LN_2	0	0	0	0

[a] Rating scale: 0 = no cells appearing alive, 4 = all cells appear alive.

tangential view of the wood, specimens were fractured with a razor blade through the vascular region. Fractured specimens were transferred quickly to fresh anhydrous ethanol, critical point dried, mounted on aluminum stubs using silver paste, and sputter-coated with gold-palladium. Specimens were observed using a JEOL JSM-840 scanning electron microscope (JEOL Ltd., Tokyo, Japan) at 10 kV accelerating voltage.

Freeze Substitution

A modified fixation procedure (2) was used to fix specimens which were frozen at time of collection. Control or frozen specimens were quench-frozen in melted Freon 12, then quickly transferred to LN_2. Specimens were transferred from LN_2 to a freeze substitution fluid consisting of 3.5% glutaraldehyde, 10 mM cacodylate buffer (pH 7.2), and 90% ethanol. Vials containing freeze substitution fluid had previously been placed in an aluminum block and allowed to cool to −80°C. After 3 d in freeze substitution fluid at −80°C, specimens were rinsed with three changes of anhydrous ethanol, and three changes of anhydrous acetone. Ethanol and acetone were cooled to −80°C prior to use. The block was placed in an insulated container and transferred to a fume hood where specimens were postfixed with 1% OsO_4 in acetone for 3 h. During this time, specimens gradually warmed to room temperature. Following postfixation, specimens were rinsed with three changes of anhydrous acetone followed by three changes of anhydrous ethanol. Specimens were fractured, critical point-dried, mounted, sputter-coated, and viewed by SEM as described above for conventionally fixed specimens.

Low-Temperature SEM

To corroborate observations made with tissues prepared by conventional fixation and freeze substitution and to attempt
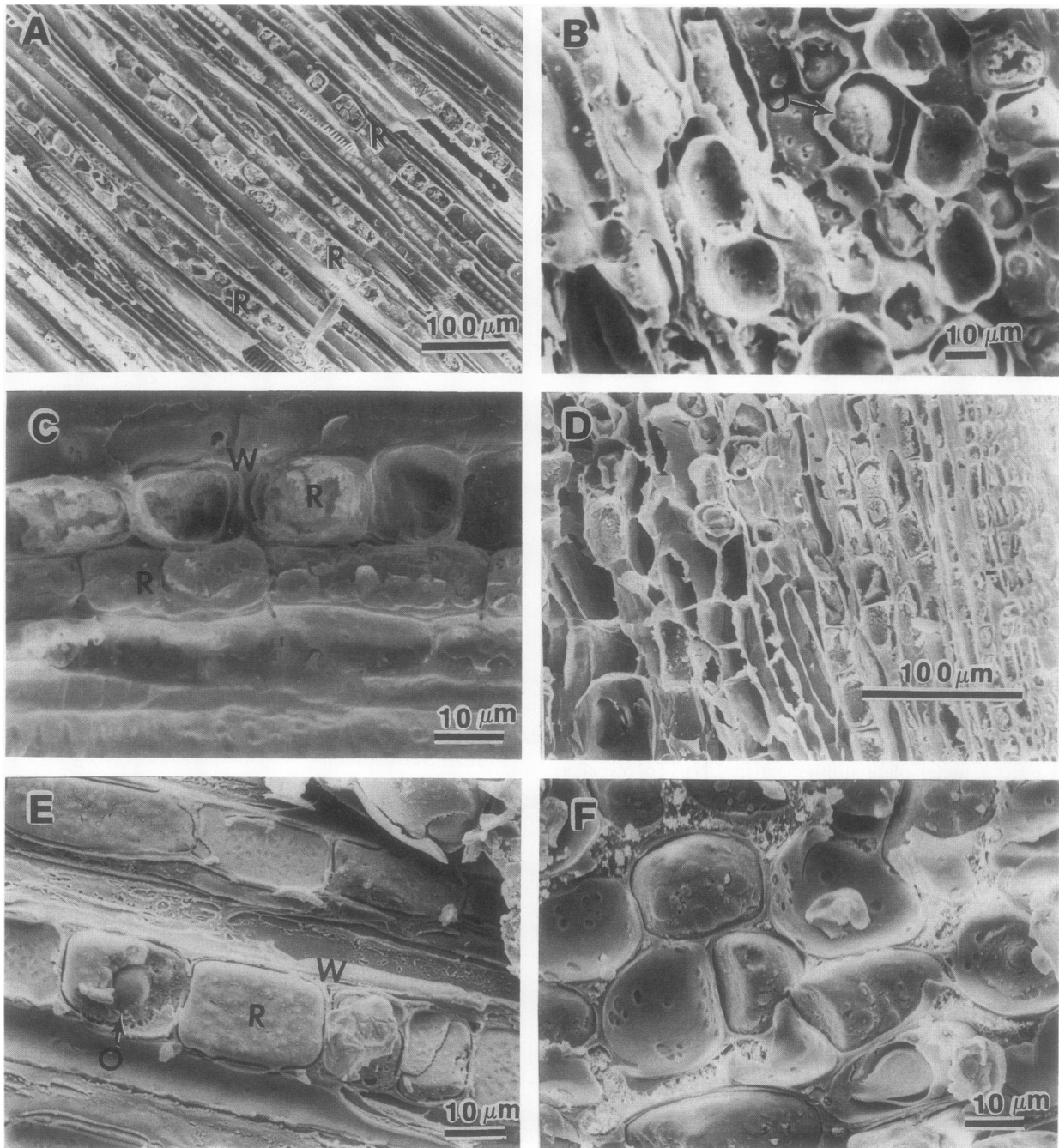
Figure 1. Examples of control specimens of wood (A, B, C) and bark (D, E, F) prepared by conventional fixation, freeze-substitution, and LTSEM. A, Flowering dogwood wood, conventional fixation; B, flowering dogwood bark, conventional fixation; C, red oak wood, freeze-substitution; D, red oak bark, freeze-substitution; E, apple wood, LTSEM; F, flowering dogwood bark, LTSEM. The three methods of preparation provided comparable preservation of cell wall location and cell contents. Note ray parenchyma cells (R), preservation of location of large organelles (O), lack of cell wall collapse (W) in ray parenchyma cells and bark cells.

to view ice *in situ,* specimens were collected for viewing under low-temperature SEM. The scanning electron microscope used was equipped with a Hexland cold stage (Hexland Ltd., East Challow, Oxfordshire, England). To facilitate transfer of freeze-stressed, quench-frozen specimens into the LTSEM unit while avoiding thawing and minimizing condensation required modification of normal cold-stage procedure. Small-diameter Ag tubes containing control or freeze-stressed specimens, which had been quench-frozen and stored in LN_2, were transferred to the SEM facility in LN_2. Tubes were loaded into holes in an aluminum disc and secured with set screws while submerged in LN_2. The aluminum disc was then loaded into the Hexland specimen holder using a transfer apparatus that could be operated while submerged in LN_2. The transfer apparatus also enabled the specimen transfer rod to be attached to the specimen holder while submerged in LN_2. This arrangement minimized the possibility of thawing and moisture contamination since the specimen was surrounded by LN_2 while it was moved from the LN_2 container to the cryochamber of the scanning electron microscope. Specimens were fractured using a scalpel blade inside the cryochamber. Specimens were moved from the cryochamber to the microscope stage and viewed at 1 or 2 kV. Specimens were viewed

without etching or were etched and sputter-coated using the sputter-coater in the cryochamber. An alterative method for preparing control specimens was to attach an unfrozen specimen to a slotted aluminum disc with a carbon-O.C.T. compound (Tissue-Tek, Miles Inc., Diagnostic Div., Elkhart, IN) cryoadhesive mixture, load the disc into the specimen holder, freeze in a nitrogen slush under vacuum, and transfer to the SEM cryochamber of the scanning electron microscope.

RESULTS AND DISCUSSION

DTA and Tissue Hardiness Tests

DTA experiments of wood specimens with bark removed confirmed previous classifications of red osier dogwood and weeping willow as nonsupercooling species. Single exotherms were detected at −6.7 and −9.5°C in red osier dogwood and weeping willow, respectively. A single exotherm was also detected in corkscrew willow at −8.8°C. LTEs were not observed in any of these species. Flowering dogwood, apple, and red oak were confirmed as species that exhibit the supercooling characteristic. Exotherms were detected in flowering dogwood at −8.6 and −40.1°C, in apple at −9.7 and −40.8°C, and in red oak at −12.3 and −40°C. Only the high-tempera-
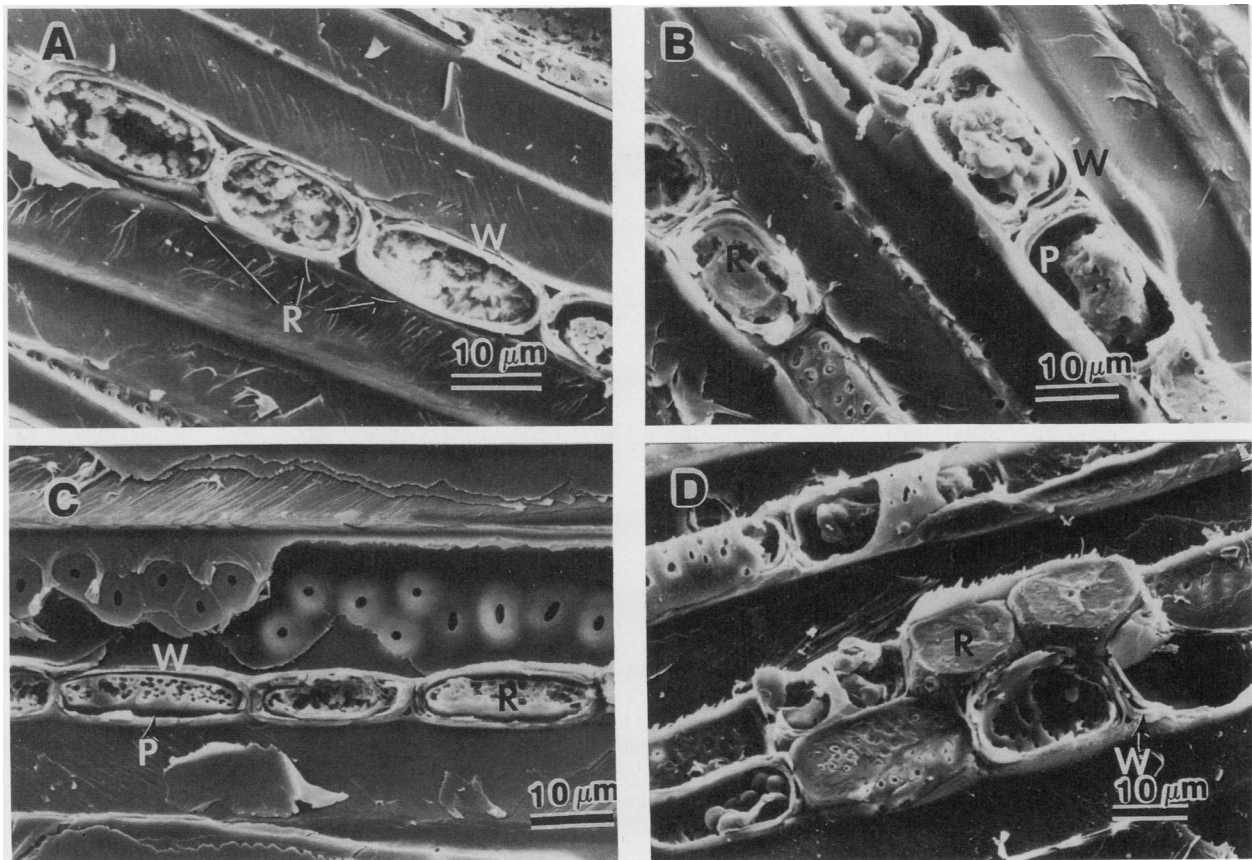
Figure 2. Wood specimens collected from the field during natural freezing events, prepared by freeze-substitution method. Note lack of cell wall collapse in ray cells (R) in both nonsupercooling (A, C) and supercooling (B, D) species. Reduction in protoplast volume (P) apparent in individual ray cells in nonsupercooling species (A, C), but cell walls (W) not collapsed. A, Corkscrew willow, collected at −5°C; B, red ash, −5°C; C, weeping willow, −10°C; D, flowering dogwood, −10°C.
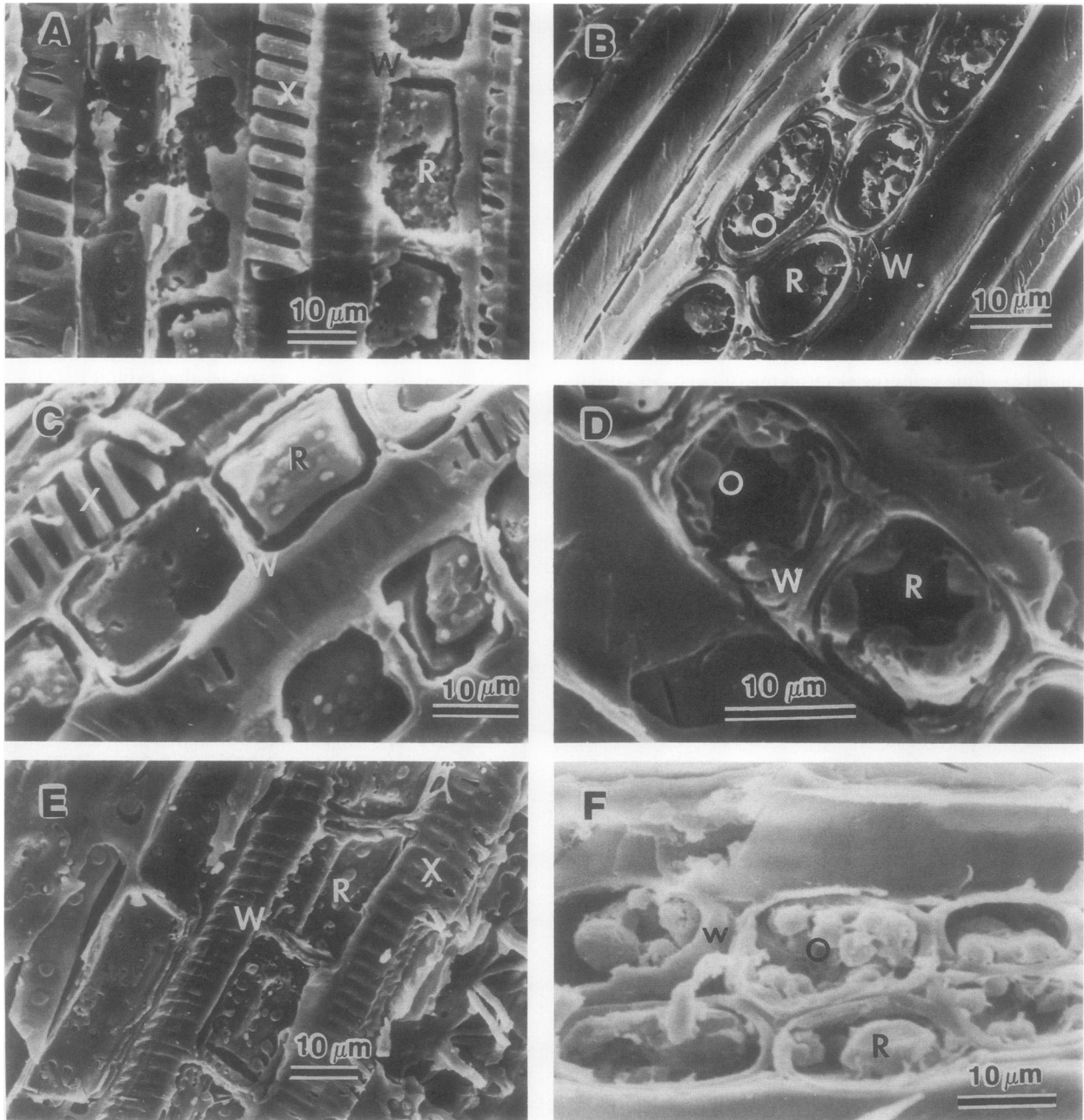
Figure 3. Wood tissue subjected to freezing stress by plate-freezing method and prepared by freeze-substitution method. Note lack of cell collapse of ray cell walls (R) in the nonsupercooling red osier dogwood (A, C, E) and the supercooling red ash (B, D, F) when subjected to freezing stress treatments of −10°C (A, B), −20°C (C, D), and −60°C (E, F). No evidence of rupturing of shared cell walls (W) or displacement of large organelles (O) in either species. These species are representative of responses observed in other species examined. Xylem vessels (X).

ture exotherm was detected in scarlet oak at −11.7°C. In red ash, the only exotherm detected occurred at −41.3°C.

Neither bark nor wood tissue of any species survived being plunged into LN₂. The LN₂ submersion treatment was done to completely kill the specimens to consistently yield a "0 = all cells dead" rating. It was previously reported (13, 23) that red osier dogwood survived LN₂ temperatures if tissues were first cooled slowly to −40°C before placing in LN₂. In these experiments, tissues were plunged directly into LN₂, and this treatment was lethal (Table I).

Ray parenchyma and bark cells of red osier dogwood survived freezing temperatures as low as −60°C (Table I). Weeping willow (Table I) tissues also survived −60°C as indicated by TZ staining. Tissue browning tests indicated that weeping willow was less hardy than red osier dogwood at exposures below −40°C. However, the TZ test was better suited to evaluation of individual cells, which was the focus of the experiments. Also, precision of the tissue browning test was limited by the original color of the wood and species variation in degree of browning. Corkscrew willow was not included in survival tests.

Species which exhibit supercooling, such as flowering dogwood, apple, red oak, scarlet oak, and red ash, were not expected to survive temperatures below −40°C. Flowering dogwood cells appear to have survived well when subjected to a freezing stress of −20°C (Table II). Survival was reduced with further cooling to −30 or −40°C, and tissues were almost completely killed by exposure to −50 or −60°C. Wood of red oak was as hardy as that of flowering dogwood, while the bark of red oak could be subjected to colder temperatures before injury occurred (Table II). Red ash (data not presented) survival results were similar to those of red oak, i.e. significant cell death occurred at and below −40°C in wood and below −40°C in bark. Few apple or scarlet oak cells (data not presented) survived −40°C, and virtually none survived at −50 or −60°C. The failure of supercooling species to exhibit significant survival below −40°C was consistent with previous reports (7, 11, 22) and coincides with results of DTA experiments.

Due to the number of species and treatments involved, it is impossible to show micrographs of all species subjected to all treatments. However, the examples presented are representative of observations made of other species subjected to identical treatments and preparation methods.

Control Specimens

Control specimens were prepared for SEM observation by several methods to confirm that comparable preservation was
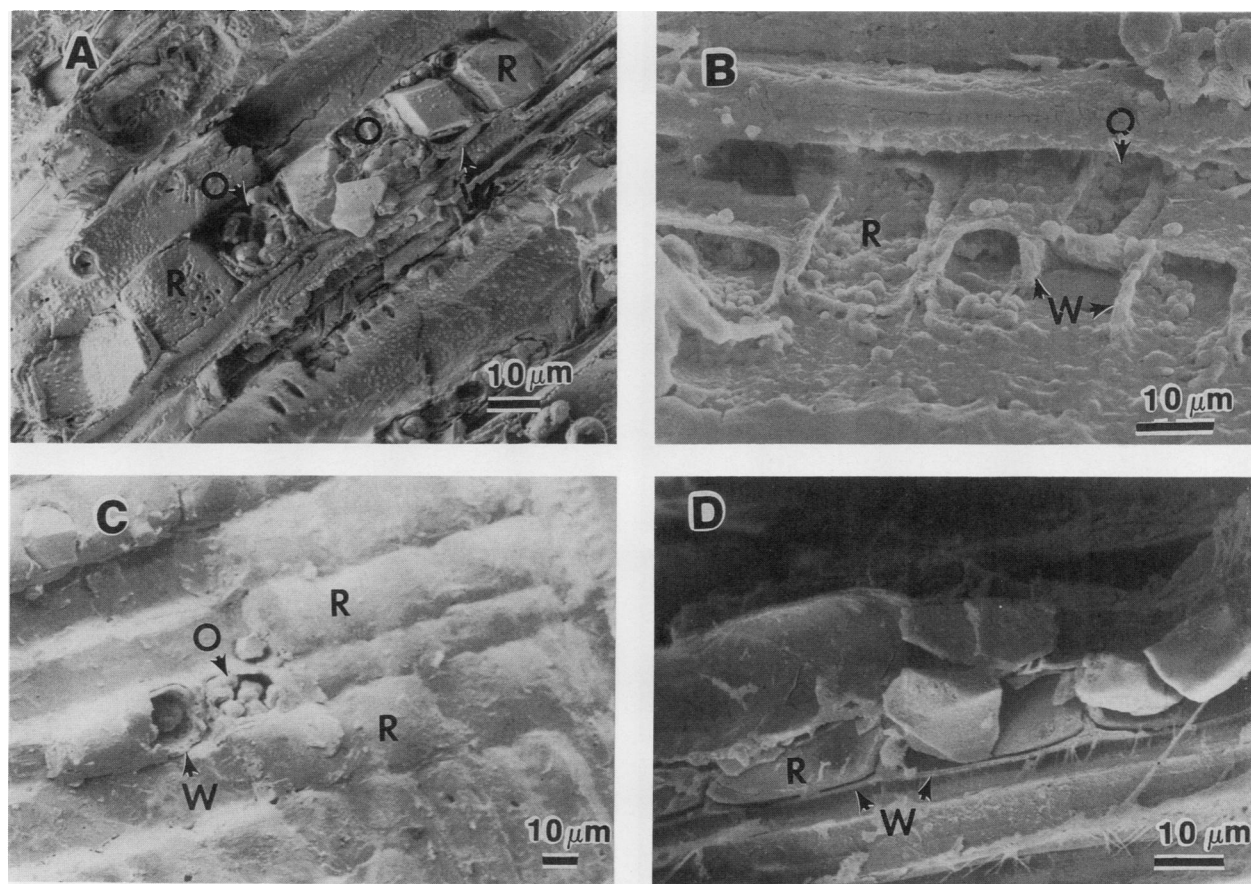
Figure 4. Examples of freeze-stressed supercooling (A, C) and nonsupercooling (B, D) species observed using LTSEM. Ray cells (R) did not collapse as a result of freezing stress of either −20 or −60°C. A, Flowering dogwood, −20°C; B, weeping willow, −20°C; C, flowering dogwood, −60°C; D, weeping willow, −60°C. Note organelles (O) in the cytoplasm, cell walls (W).
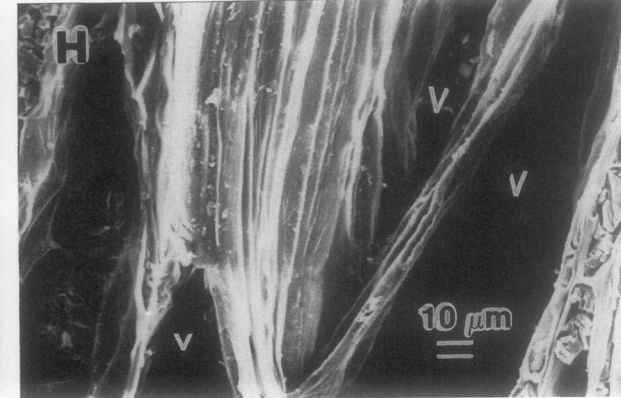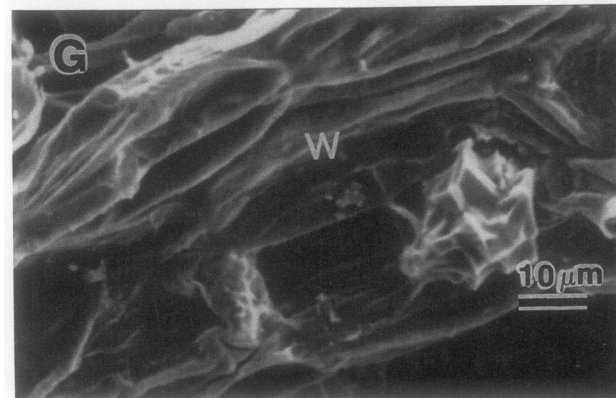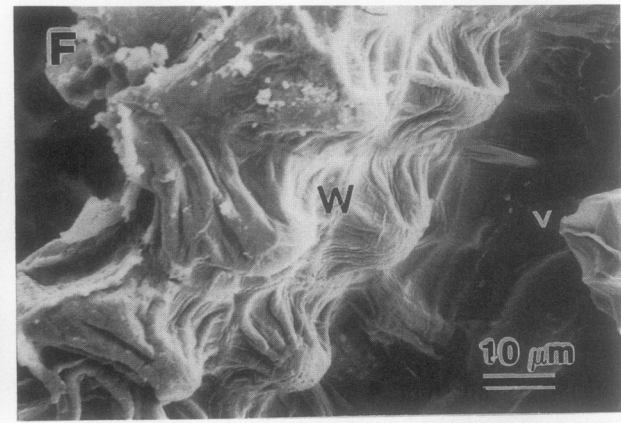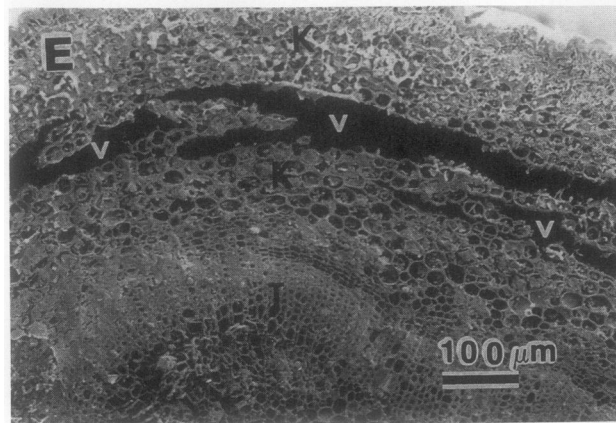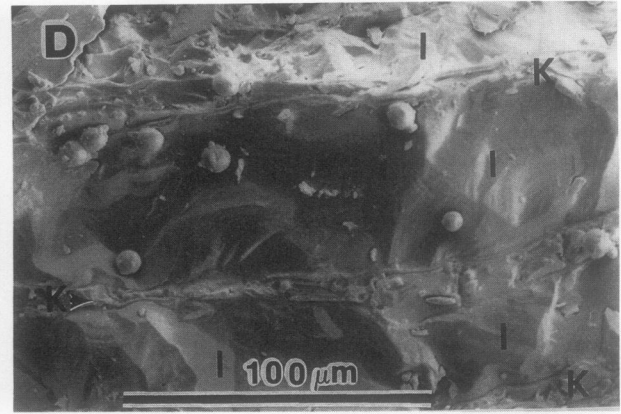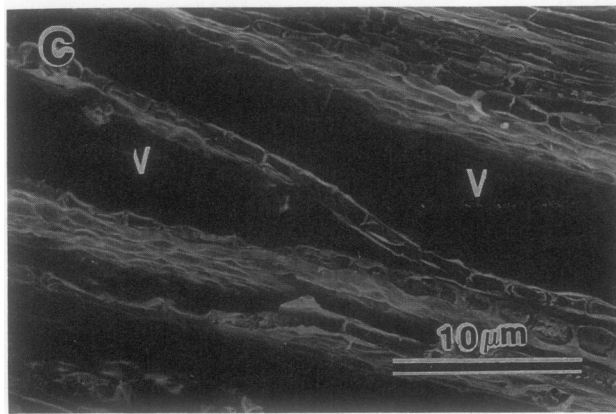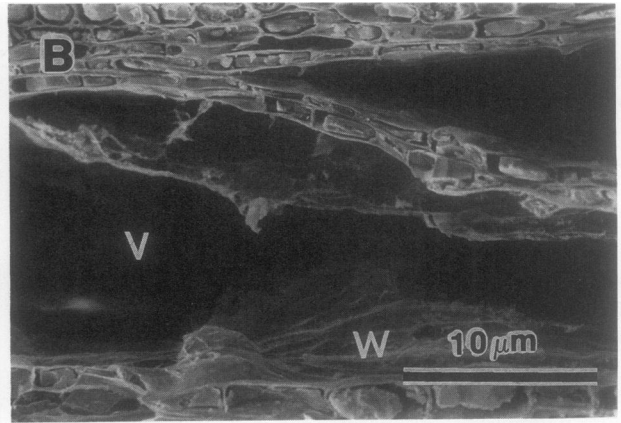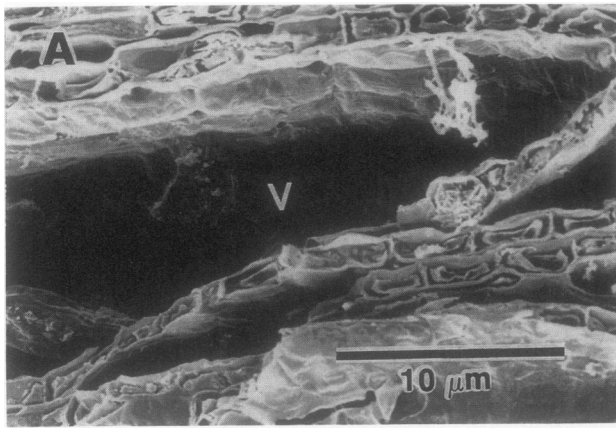
obtained using conventional fixation, freeze substitution, and LTSEM. This was important to the identification of potential artifacts and to establish the legitimacy of the freeze substitution method for preserving cell wall, membrane, and intracellular constituents. Examples of control specimens of wood and bark prepared by the three different methods are presented in Figure 1. Conventional fixation (Fig. 1A, wood, and 1B, bark) was used as a standard, since it is a commonly used procedure for SEM. Fixation of woody tissues by freeze substitution (Fig. 1C, wood, and 1D, bark) resulted in adequate preservation for the purposes of these experiments. Comparison with conventionally fixed specimens indicated that there were no apparent artifacts induced by the freeze substitution procedure, such as alterations in cell volume or displacement of large organelles in ray parenchyma or in bark tissues. Comparison of freeze-substituted and conventionally fixed controls with LTSEM controls (Fig. 1, E and F) provided additional confirmation. Classification as supercooling or nonsupercooling should not be a factor in the capability of these preservation methods to preserve plant tissues. No differences in quality of preservation were observed between any of the eight species examined.

Field Frozen Specimens

Cell collapse was not seen in ray parenchyma cells of supercooling or nonsupercooling species in specimens collected from the field during exposure to freezing temperatures of −5, −10 (Fig. 2), or −15°C (data not presented) and prepared by freeze substitution. Cell collapse was interpreted to be a distinct response in which cell walls appeared to be imploded with a distorted, wrinkled appearance as they shrank around the dehydrated protoplast; such as described in bark, herbaceous plants, and animal cells (2, 17–19, 24). The lack of cell collapse noted in the xylem ray cells was expected in supercooling species (2, 15, 16, 21, 22) such as red ash (Fig. 2B), flowering dogwood (Fig. 2D), apple, red oak, and scarlet oak (data not presented). Cell collapse was expected in nonsupercooling species (17–19, 24) such as corkscrew willow (Fig. 2A), and weeping willow (Fig. 2C), and red osier dogwood (data not presented). Cell collapse as a result of extracellular ice formation was expected in the nonsupercooling species since no LTE was detected and these species survived exposures below −40°C. However, cell collapse of xylem ray cells was not observed in any of the three nonsupercooling species frozen in the field.

Specimens Prepared by Laboratory Freezing Protocol

Freezing stresses imposed using the plate freezing technique were used to simulate natural freezing stress, and to expose tissues to temperatures colder than experienced in our area. Examples of ray parenchyma cells from specimens stressed at −10 (Fig. 3, A and B), −20 (Fig. 3, C and D), and −60°C (Fig. 3, D and E) were presented to compare a nonsupercooling (red osier dogwood) and a supercooling (red ash) species at three levels of freezing stress. Other species responded similarly. As in the field-frozen specimens and unfrozen controls, no cell collapse was observed in ray parenchyma cells from either the supercooling or nonsupercooling species. Even when exposed to −60°C stress, no collapse of ray parenchyma cell walls was observed in any species examined.

Possible criticisms of the plate freezing technique relate to the cooling rate and specimen size. The cooling rate of 5°C/h was more rapid than usually occurs in nature. We do not believe this resulted in artifacts since similar results were observed with both plate frozen and field frozen specimens. Small specimens were used to facilitate penetration of fixatives, and this could have resulted in sample drying or altered freezing behavior. The tendency of specimens to dry during the freezing procedure was minimized by coating the plate with vegetable oil and keeping specimens covered. Observations made using specimens frozen to −5 and −10°C by the plate freezing methods and specimens collected in the field were similar. Therefore, the suitability of the plate freezing method to provide a freezing stress representative of field conditions was established (Figs. 2 and 3).

No cell collapse was observed in LTSEM preparations of both supercooling and nonsupercooling specimens stressed at −20 or −60°C (Fig. 4). Ray parenchyma cells appeared full size in both types of species. This confirmed our earlier observations of both field frozen and laboratory frozen specimens prepared by freeze substitution. A possible criticism of the freeze substitution procedure was that it failed to preserve tissue morphology, and that cells that collapsed during freezing returned to full size during fixation. This was not a problem with LTSEM because chemical fixation was not used. Tissues were quench frozen so that all cell constituents were frozen in place and the specimen loading/transfer technique minimized the possibility of movement of cell parts due to thawing or recrystallization.

It could be argued that the reason cell wall collapse was not observed was that the walls returned to their prefrozen position as ice was removed during the freeze substitution procedure. If this were the case, evidence of distorted cell walls, separation of neighboring cells, or rupturing of shared walls would be expected. These were not observed in ray cells of any species examined (Figs. 2–4). Due to the extensive cell-to-cell connections in tissue regions where ray parenchyma cells are found, it would be difficult to imagine that cell wall collapse would have occurred without severe tearing and

---

Figure 5. Bark tissues of red osier dogwood (A, C, D) and flowering dogwood (B, E) subjected to freezing stress by plate-freezing method. Note presence of ice voids (V) and collapsed cell walls (W) adjacent to voids in red osier dogwood collected at −10°C (A), flowering dogwood at −20°C (B), and red osier dogwood at −60°C (C). Ice crystals (I) are visible in red osier dogwood bark collected at −20°C when observed using LTSEM (D) and typical of ice crystals which caused voids in freeze-stressed bark (A, B, C). Note collapsed bark cells (K) between and adjacent to ice crystals. Several voids noted in outer bark of freeze-stressed flowering dogwood (E). Voids appeared as a ring in the bark and were observed in other species. Vascular tissue (T) is to the lower left. Bark tissues from weeping willow (F), corkscrew willow (G), and flowering dogwood (H) collected at −10°C during a natural freeze. Collapsed cell walls (W) adjacent to an ice void appeared shriveled. Voids (V) and collapsed cell walls (W) observed in bark of both plate-frozen and field-frozen specimens were similar.

disruption of ray parenchyma cell walls as well as walls of adjacent vascular cells.

In some instances, individual ray parenchyma cells appeared to have a reduced volume of protoplasm. However, this apparent reduction in protoplast volume was not accompanied by cell wall collapse (Fig. 3, C and F). If a reduction in protoplast volume occurred during freezing, it represented a distinct response. Separation of the protoplast from the cell wall was observed in both supercooling and nonsupercooling species, and occurred in individual cells, not in extended groups of cells. In many cases, a single ray cell appeared to have reduced protoplast volume, yet was situated between other ray cells which did not. It appeared that the number of xylem ray parenchyma exhibiting this condition increased at decreasing temperatures; however, this was not quantified. That this was observed in nonsupercooling species which survived exposures as low as −60°C indicated this response was not lethal. A possible explanation for the reduction of protoplast volume was that intracellular water moved from the cell to extracellular ice crystals. The location of such ice crystals was not apparent, but ice may have been located in nearby xylem vessels. Another possible explanation was that small ice crystals formed in the space between the cell wall and the plasma membrane. Further experiments would be required to determine the location of ice crystals and their association with response of particular cells in these tissues.

Bark

In contrast to xylem ray parenchyma, cell collapse was observed in bark tissues subjected to freezing stress (Fig. 5). Voids were observed in bark of freeze substitution prepared specimens. Voids appeared to occur between layers of bark cells and were thought to be the result of ice crystal growth. Ice was replaced by the fluids used in the fixation procedure and voids were preserved in place because fixation took place well below the melting temperature of water. Similar voids have been observed in apple bark (2) and peach bud scales (1). Figure 5D shows an ice crystal separating layers of bark in flowering dogwood frozen to −15°C. The size and location of this ice crystal was representative of those observed in all

species and confirms the association of ice crystals with voids observed in freeze-stressed specimens prepared by freeze substitution. Voids were generally found in the portion of the bark outside the cambium, the outer edge of the void located 6 to 10 cells in from the epidermal layer (Fig. 5E). When viewed in cross-section, voids formed a discontinuous ring which appeared to separate the outermost layers of bark from the inner layers. The number and size of voids appeared to increase in all species at lower temperatures. Voids formed during natural freezing episodes persisted and were observed in bark collected in early spring (data not presented). Numerous cells adjacent to voids were collapsed (Fig. 5, A, B, and C). Cell collapse adjacent to voids indicated that bark cells froze extracellularly. Collapsed cells walls were shriveled and disorganized (Fig. 5F). Cell walls that appeared to have been near cell-to-cell connections were often ruptured, and this was particularly noticeable near the ends of ice voids. Cell walls closer to the middle of the voids were less likely to be torn but had a collapsed appearance. Both supercooling (Fig. 5B) and nonsupercooling (Fig. 5, A and C) species exhibited this response in bark tissues.

Voids were also observed in bark of supercooling (Fig. 5G) and nonsupercooling (Fig. 5H) species that were collected outdoors during a natural freeze. Collapsed cells adjacent to voids were also observed. The similarity in response of bark cells from field and plate frozen specimens was another indication that the plate freezing method was adequate for imposing freezing stress (cf. Fig. 5, A, B, C with Fig. 5, G, H).

Pith

Pith cells did not appear to be affected by freezing stress (Fig. 6). Pith cells from both controls and freeze-stressed specimens were indistinguishable in the species observed. These results were not surprising since specimens were taken from plants that were cold-acclimated prior to collection. Furthermore, pith cells have been reported to exhibit LTEs, which would indicate that they supercool, so cell collapse would not be expected (2, 7, 16, 20, 22). Our observations did not disagree with that prediction. Pith cells from both control and freeze-stressed specimens were highly vacuolated,
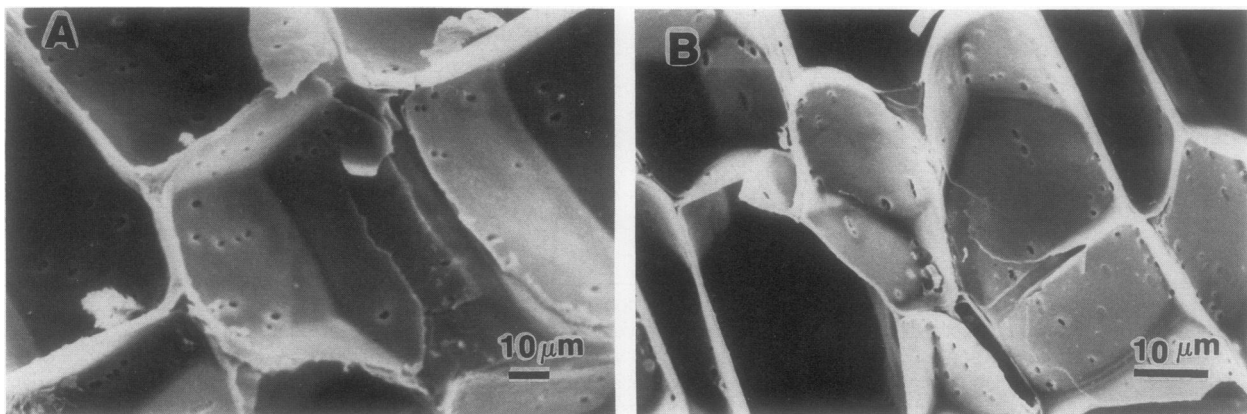
Figure 6. Pith cells did not appear to be altered by freezing stress in any species. A, weeping willow control pith; B, red osier dogwood pith freeze-stressed to −20°C.

and plasma membranes were tightly pressed against the cell wall.

CONCLUSION

In summary, bark of both classes of woody plants exhibited extracellular freezing. Cells collapsed and voids resulting from the presence of extracellular ice crystals were observed in freeze-stressed specimens. Identical observations were made with specimens frozen in the field and in the laboratory. The association of ice crystals with voids in outer bark layers was confirmed by LTSEM.

Cell collapse was not observed in ray parenchyma of the supercooling species flowering dogwood, apple, red oak, and scarlet oak. Hardiness tests and DTA confirmed predicted survival and exotherm characteristics. Results were consistent with the deep supercooling classification.

Cell collapse was not observed in ray parenchyma cells of freeze-stressed specimens from the nonsupercooling woody species red osier dogwood, weeping willow, or corkscrew willow when frozen to temperatures as low as −60°C. This was observed both in specimens prepared for SEM by freeze substitution, and in LTSEM specimens, and observed in response to both a laboratory freezing protocol and under natural freezing conditions in field. That two different preparation methods yielded consistent results confirmed that the lack of cell collapse was not an artifact of either protocol. If lack of cell collapse had been a result of cells returning to prefrozen positions during freeze substitution, it would still have been observable with LTSEM. The legitimacy of the plate freezing method to simulate natural freezing conditions was confirmed by the similarity of results from both methods. The possibility that cell collapse was overlooked was minimized by observing cells fractured in various planes. Hardiness and DTA results indicated that these species survived −60°C exposure and did not supercool. Lack of cell collapse was inconsistent with the predicted response that ray parenchyma cells of cold-hardy, nonsupercooling species freeze extracellularly. These cells did not collapse, yet survived temperatures below the homogeneous nucleation temperature. Ray parenchyma cells of red osier dogwood, weeping willow, and corkscrew willow do not fit well into either the supercooling or nonsupercooling categories. Therefore, alternative explanations of their freezing behavior are required.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Ashworth EN, Davis GA, Wisniewski ME (1989) The formation and distribution of ice within dormant and deacclimated peach flower buds. Plant Cell Environ 12: 521–528

2. Ashworth EN, Echlin P, Pearce RS, Hayes TL (1988) Ice formation and tissue response in apple twigs. Plant Cell Environ 11: 703–710

3. Ashworth EN, Rowse DJ, Billmyer LA (1983) The freezing of water in woody tissues of apricot and peach and the relationship to freezing injury. J Am Soc Hortic Sci 108: 299–303

4. Becwar MR, Rajashekar C, Hansen-Bristow KJ, Burke MJ (1981) Deep undercooling of tissue water and winter hardiness limitations in timberline flora. Plant Physiol 68: 111–114

5. Berlyn GP, Miksche JP (1976) Botanical Microtechnique and Cytochemistry. Iowa State University Press, Ames, pp 33–34

6. Burke MJ, Bryant RG, Weiser CJ (1974) Nuclear magnetic resonance of water in cold acclimating red osier dogwood stem. Plant Physiol 54: 392–398

7. Burke MJ, Gusta LV, Quamme HA, Weiser CJ, Li PH (1976) Freezing and injury in plants. Annu Rev Plant Physiol 27: 507–528

8. Burke MJ, Stushnoff C (1979) Frost hardiness: a discussion of possible molecular causes of injury with particular reference to deep supercooling of water. In H Mussell, RC Staples, eds, Stress Physiology of Crop Plants. John Wiley & Sons, New York, pp 199–225

9. George MF, Becwar MR, Burke MJ (1982) Freezing avoidance by deep undercooling of tissue water in winter-hardy plants. Cryobiology 19: 628–639

10. George MF, Burke MJ (1977) Cold hardiness and deep supercooling in xylem of shagbark hickory. Plant Physiol 59: 319–325

11. George MF, Burke MJ, Pellett HM, Johnson AG (1974) Low temperature exotherms and woody plant distribution. HortScience 9: 519–522

12. Gusta LV, Tyler NJ, Chen THH (1983) Freezing of water in red-osier dogwood stems in relation to cold hardiness. Plant Physiol 72: 122–128

13. Guy CL, Niemi KL, Fennell A, Carter JV (1986) Survival of Cornus sericea L. stem cortical cells following immersion in liquid helium. Plant Cell Environ 9: 447–450

14. Harrison LC, Weiser CJ, Burke MJ (1978) Freezing of water in red-osier dogwood stems in relation to cold hardiness. Plant Physiol 62: 899–901

15. Hong SG, Sucoff E (1980) Units of freezing of deep supercooled water in woody xylem. Plant Physiol 66: 40–45

16. Hong SG, Sucoff E, Lee-Stadelmann O (1980) Effect of freezing deep supercooled water on the viability of ray cells. Bot Gaz 141: 464–468

17. Lovelock J (1953) Haemolysis of human red blood cells by freezing and thawing. Biochim Biophys Acta 10: 414–426

18. Mazur P (1969) Freezing injury in plants. Annu Rev Plant Physiol 20: 419–448

19. Meryman HT (1971) Osmotic stress as a mechanism of freezing injury. Cryobiology 8: 489–500

20. Quamme HA, Gusta LV, Chen PM (1982) Relationship of deep supercooling and dehydration resistance to freezing injury in dormant stem tissues of "Starkrimson Delicious" apple and "Siberian C" peach. J Am Soc Hortic Sci 107: 299–304

21. Quamme H, Stushnoff C, Weiser CJ (1973) The mechanism of freezing injury in xylem of winter apple twigs. Plant Physiol 51: 273–277

22. Quamme HA, Weiser CJ, Stushnoff C (1972) The relationship of exotherms to cold injury in apple stem tissues. J Am Soc Hortic Sci 97: 608–613

23. Sakai A (1960) Survival of the twig of woody plants at −196°C. Nature 185: 393–394

24. Siminovitch D, Scarth GW (1938) A study of the mechanism of frost injury to plants. Can J Res Ser C 16: 467–481

25. Steponkus PL, Lanphear FD (1967) Refinement of the triphenyl tetrazolium chloride method of determining cold injury. Plant Physiol 42: 1423–1426