Short Communication

Supercooling in Overwintering Azalea Flower Buds

ADDITIONAL FREEZING PARAMETERS

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ABSTRACT

Results of calorimetric, nuclear magnetic resonance, and low temperature light microscopic studies on supercooled azalea (Rhododendron kosterianum, Schneid.) floral primordia are reported. Heat release during freezing of the supercooled floral primordia is in the range predicted for supercooled pure water. Spin-lattice and spin-spin relaxation times measured by pulsed nuclear magnetic resonance spectroscopy decreased after freezing, suggesting that a redistribution of tissue water is associated with injury to the floral primordium. The calorimetric and low temperature microscopy studies showed no detectable ice formation in floral primordia until the major freezing event at low temperature. No resistance to ice growth is found to exist in the primordium tissues, indicating that a freezing barrier or thermodynamic equilibrium exists between the unfrozen primordium and other flower bud parts which contain ice at subfreezing temperatures.

Differential thermal analysis and continuous wave NMR spectroscopy experiments have shown that floral primordia in overwintering azalea flower buds supercool to low temperatures and, therefore, avoid freezing injury (5). Recent evidence indicates that this phenomenon also occurs in several Prunus species (10). The finding that a number of cold-hardy woody species native to the eastern deciduous forest have low temperature freezing points in the xylem near the homogeneous nucleation temperature of water (−38 C) suggests that supercooling may be a more important freezing resistance mechanism than originally presumed (4, 11). In the present study, some additional freezing parameters for azalea floral primordia are measured using DSC and pulsed NMR. Results of low temperature microscopy experiments are also discussed.

MATERIALS AND METHODS

DSC and pulsed NMR experiments were performed on primordia excised from whole flower buds of deciduous azalea. Low temperature microscopy was carried out on primordium sepals.

Plant Materials. Flower buds were collected April 11, 1975 from plantings of deciduous azalea (Rhododendron kosterianum, Schneid.) on the St. Paul campus of the University of Minnesota. Buds were stored at 5 C in stoppered flasks until tested. All tests were completed within 7 days.

NMR Spectroscopy. Pulsed NMR experiments were performed at 20 MHz on a Bruker M-20 pulsed spectrometer with a programmable temperature controller. Seventeen excised primordia were weighed and placed in a single NMR tube. Liquid water content of the primordium was measured at 2 C and −35 C by monitoring the free induction decay after the second and succeeding pulses of a 90 degree pulse series, each pulse separated by 1 sec. The free induction decay was monitored 6 μsec after the pulse center. Samples were cooled from 2 C to −35 C at 1 C/min. After testing, primordia were dried in a vacuum oven at 70 C for at least 2 days. Water content/g dry weight was evaluated and NMR signal from the dry sample was recorded. Correction for NMR signal from ice and dry sample was made as described by Burke et al. (2). Boltzmann temperature correction was also made. T1 (the spin-lattice relaxation time) and T2 (spin-spin relaxation time) of tissue water were evaluated before and after freezing by the method of Burke et al. (1) for similar relaxation measurements on red osier dogwood stems.

DSC. Individual excised floral primordia were used and tests were performed on a Perkin-Elmer DSC-2 differential scanning calorimeter. Two experiments were conducted. In the first, heat release (ΔQ) during freezing was measured for three primordia. The cooling rate was 1.25 C/min and temperature of supercooling for each primordium was recorded. After testing, primordia were dried in a vacuum oven at 70 C to determine total tissue water. ΔQ/g H2O was calculated by dividing the heat measured experimentally by the fraction of total H2O freezing between 0 and −35 C as determined by NMR. ΔQ/g H2O for a primordium was compared with heat of fusion for supercooled H2O at the primordium freezing temperature. To determine if any freezing of water occurred prior to the major freezing event, a primordium was cooled at 0.31 C/min on the most sensitive calorimeter scale (0.1 mcal/sec). Instrumental noise level of the DSC-2 calorimeter is approximately of the order of heat released during spontaneous crystallization of H2O in 10 floral primordium cells. The diameter of a dormant Rhododendron floral primordium cell is approximately 20 × 10⁻³ cm (13).

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4 Abbreviations: NMR: nuclear magnetic resonance; DSC: differential scanning calorimetry; gH2O/g dw: water content/gram dry weight; ΔQ: heat release.
Low Temperature Microscopy. Microscopy was performed on a Wild M20 microscope with low temperature stage constructed as described by George and Burke (3). Sepals were removed from excised primordia and mounted in either fluorocarbon liquid (FC-80, Minnesota Mining and Manufacturing Company) or in spring water. Fluorocarbons have been used previously for testing of plant tissues (15). When specimens were mounted in water, they were nucleated near 0°C by freezing a thin film of water which extended from outside the freezing stage to the tissue. Observation of the tissues at a particular subfreezing temperature was accomplished by cooling at 2 ± 1°C/min to the temperature and then holding. This procedure was repeated until the tissue froze.

RESULTS AND DISCUSSION

Water content for the primordia used in the NMR experiments was 1.34 g H2O/g dry weight as determined by drying the tissue in a vacuum oven. Liquid water unfrozen at −35°C determined by pulsed NMR was 0.31 g H2O/g dry weight. This value is in the range of unfrozen water at similar low temperatures for red osier dogwood stems (1), crowns of winter wheat (7), and frozen protein and polypeptide solutions (8, 9). The fraction of water freezing between 0 and −35°C is, therefore, 0.77. This value is used below for analysis of ΔQ/g H2O at the primordium freezing point.

Pulsed NMR measurements of T1 and T2 made on the primordia before and after freezing to −35°C are given in Table I. T1 demonstrated a fast and slow component, the slow component accounting for approximately 60% of the sample water. Within the errors of measurement, T2 could only be resolved into one component. Multicomponent systems such as biological samples often have several groups of protons which may or may not be averaged by an exchange process (2). When the relaxation times of several groups are significantly different, T1 and T2 may be resolved into separate components. Acclimated stems of red osier dogwood (1) and shagbark hickory (3) have been shown to display slow and fast components for both T1 and T2. T1 and the slow T1 component of primordium water demonstrate marked decrease after freezing to −35°C (Table I). This result is in agreement with continuous wave NMR experiments which showed an approximate 2-fold increase in NMR linewidth after a primordium freezes (4). NMR linewidth at one-half peak height is reciprocally related to T2 (1). Schneider (13) has observed that dormant Rhododendron primordia have small intercellular spaces and large vacuoles. Most tissue water is, therefore, intracellular. Rhododendron primordia also contain segregated protein bodies and amyloplasts in epidermal and mesophyll cells (13). The sudden freezing of supercooled cellular contents at low temperature could conceivably lead to a dispersion of these macromolecules in the vacuole. This may, in part, cause the decrease in T1 and T2 relaxation times. Earlier work has shown that a primordium is killed when it freezes (6). The decrease in relaxation times of cellular water is, therefore, a direct indication of freezing damage to the primordium.

Measurements of heat release during freezing for the three primordia tested are in good agreement with predicted heats of fusion for supercooled water at the same freezing temperatures (Table II). Determination of heat of fusion for supercooled water was made using data of Rasmussen and Mackenzie (12).

<table>
<thead>
<tr>
<th>Primordium</th>
<th>Freezing Temperature (C)</th>
<th>ΔQ/g H2O (cal/g)</th>
<th>ΔH Fusion for Pure Water (cal/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−16.0</td>
<td>71.3</td>
<td>71.4</td>
</tr>
<tr>
<td>2</td>
<td>−16.0</td>
<td>64.8</td>
<td>72.0</td>
</tr>
<tr>
<td>3</td>
<td>−17.5</td>
<td>65.3</td>
<td>71.1</td>
</tr>
</tbody>
</table>

1 from data of Rasmussen and Mackenzie (12)

The results support the contention that floral primordia resist low temperature injury in winter by remaining supercooled and that the heat release or low temperature exotherms result from freezing of cellular water (5). Cooling a primordium at 0.31°C/min on the highest sensitivity setting (0.1 μcal/sec) of the scanning calorimeter showed that no detectable water froze until the major freezing event at −26.1°C.

Microscopic analysis on primordial sepal mounted in fluorocarbon showed that ice formation began at a single locus and quickly spread throughout the entire sepal. Ice crystallization appeared to be intracellular as the cells were observed to darken rapidly as the ice front moved through the tissue. The lowest temperature recorded for a sepal freezing on the microscope stage was −16°C. The necessity of removing a part of the primordium for microscopic observation may produce undetermined changes in the freezing pattern. However, DSC analysis indicates that the sepal freezes near the same temperature that the whole primordium freezes, and differential thermal analysis of seasonal bud hardiness (5) has shown that primordia freezing points can be expected to vary over the range observed in the DSC and microscopic experiments. Therefore, there is justification for the legitimacy of the microscopic observations. When sepal were mounted in spring water and the water was seeded with ice crystals slightly below 0°C, the tissue was observed to begin freezing before −5°C. Ice growth appeared to begin in areas of the sepal which were injured during removal from the primordium and then spread slowly throughout the entire sepal. Although the small cell size prevented close observation, freezing was probably in part extracellular. This result indicates that if an ice crystal is introduced into a primordium slightly below 0°C, no resistance to ice growth exists in the primordium tissue itself. DSC experiments demonstrate that no ice formation takes place in an excised floral primordium prior to the major freezing event. Since in the whole azalea flower bud ice does exist in the stem axis and bud scales above −10°C (5, 6), a physical barrier or a thermodynamic equilibrium must exist between these tissues and the floral primordium. The barrier or equilibrium is required to prevent direct ice growth into the primordium and to prevent sublimation of the supercooled cellular water inside the primordium cells to the extraprimordium ice. Freezing injury to floral primordia in winter cereals has been shown to depend to some extent on the ability of vascular tissue to stop the advance of the freezing boundary (14). It has been suggested (3) that the ability of xylem ray parenchyma in shagbark hickory to remain supercooled at subfreezing temperatures is due to simple surface chemical effects which allow tissue water to remain in equilibrium with ice outside of the tissue. Further analysis will be required to understand the nature of the resistance and morphological details which allow one part of the flower bud to remain supercooled when tissues only short distances away are frozen.

DSC, pulsed NMR, and low temperature microscopy observations presented here add further evidence supporting the conclusion that overwintering azalea primordia survive low temperature in winter by remaining in a supercooled state.
LITERATURE CITED


