Determination of Unfrozen Water in Winter Cereals at Subfreezing Temperatures

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ABSTRACT

The freezing of water in acclimated and nonacclimated cereals was studied using pulsed nuclear magnetic resonance spectroscopy. The quantity of unfreezable water per unit dry matter was not strongly dependent on the degree of cold acclimation. In contrast, the fraction of water frozen which was tolerated by nonacclimated winter cereals and by an acclimated spring wheat (*Triticum aestivum* L.) was less than in acclimated hardy cereals. The freezing curves had the following form:

\[ L_T = L_0 \Delta T m / T + K \]

where \( L_T \) and \( L_0 \) are liquid water per unit dry matter at \( T \) and 0 C, respectively, \( \Delta T m \) is the melting point depression and \( K \) is the liquid water which does not freeze.

The freezing of tissue water and the amount frozen at the killing temperature must be important in understanding cold hardiness in plants. When a plant freezes, ice formation is normally restricted to the extracellular spaces. Liquid water is removed from the cells and frozen extracellularly, causing cell contraction. Levitt (10) suggests that an increase in hardness could result from an increased ability of the protoplasm to withstand dehydration or from an increased cell sap concentration, limiting ice formation and cell dehydration. Johansson (4) and Johansson and Krull (5) used calorimetry to study freezing of water in winter cereals of varying hardiness. They concluded that increases in hardness did not result from increased ability of the protoplasm to withstand dehydration. Rather, they suggested that the increase in hardness resulted from increased cell sap concentration decreasing cell dehydration at low temperatures. They observed that wheat plants, regardless of hardness, were killed when more than 85% of the freezable water was frozen. Ideas of a freezing or dehydration limit are not restricted to winter cereals. Williams and Meryman (15) observed that spinach grana are injured at 64% cell dehydration and the cells of several animal species have the same dehydration limit.

Determinations of ice formation in the plant generally involve cryoscopic, plasmolytic, or calorimetric methods. Levitt (9) in 1939 used the cryoscopic and plasmolytic methods to determine the freezing point lowerings of the cell sap, then assumed ideal freezing behavior. Ideal freezing behavior described here implies the absence of eutectics, the formation of pure ice during freezing, and the concentration of ideal solute in the liquid phase. Then the freezing curve predicted by plotting the liquid water against temperature is a hyperbola. Johansson (4) made extensive use of calorimetric methods to study the freezing process. He also assumed the hyperbolic form for the freezing curve and restricted his measurements of liquid water to two temperatures, −2.65 and −9 C.

Nuclear magnetic resonance has frequently been used to determine the liquid water content of partially frozen samples (1, 2, 6-8, 11). In this study, pulsed NMR spectroscopy is used to study the freezing process in crown tissue from a spring wheat, two winter wheats, and a hardy fall rye. The relationship of the freezing process to the killing temperature and the mathematical form of the freezing curve are described.

MATERIALS AND METHODS

Seeds of three cultivars of wheat (*Triticum aestivum* L.), namely Manitou spring wheat, Cappelle-Desprez and Kharkov winter wheat, and Frontier fall rye (*Secale cereale* L.) were germinated at 15 C in a soil-sand-peat mixture (2:1:1). At the three leaf stage, plants were cold acclimated by the following temperature (light-period temperature/dark-period temperature) and photoperiod regimes: 10/8 C, 16 hr for the first 7 days, 8/5 C, 16 hr for the next 7 days, 5/3 C, 14 hr for the next 7 days, and 5/1 to 0 C, 12 hr for the following 21 days. The last 7 days of hardening included a frost of −2 to −3 C for 4 hr during the dark period. This cold hardening regime acclimates crowns of Manitou to −5.5 ± 0.5 C, Cappelle-Desprez to −11 ± 1 C, Kharkov to −18 ± 1 C, and Frontier to −24 ± 1 C. Hardiness was determined by slowly cooling crowns at −2 C/hr. Ten crowns were used per test temperature spaced at 2 C intervals. After 3 weeks of 20 C, 24-hr photoperiod the crowns were rated for viability by regrowth of roots and leaves. One series of Kharkov plants was hardened for 14 days as described above for the first 14 days of hardening regime. This resulted in a hardening of −9 ± 1 C. Tender plants maintained at 20 C with a 16-hr photoperiod can survive −2 C for Manitou.

1 Abbreviation: NMR: nuclear magnetic resonance.
RESULTS AND DISCUSSION

In Figures 1 to 4, freezing curves for crowns of several tender and hardy cereals are given. The plots of liquid water per unit dry matter (L_T) versus T and 1/T have the form:

\[ L_T = L_0 \Delta Tm/T + K \] (1)

L_0 is the liquid water content before freezing. \( \Delta Tm \) and K are arbitrary parameters. A straight line is obtained when plotting \( L_T \) versus 1/T with slope \( L_0 \Delta Tm \) and intercept, K (Figs. 1–4). The fit to a straight line is good as is indicated by the values of the sample correlation coefficients given in Table I. If it is assumed that there are no eutectics and that only pure ice is formed during freezing, then \( \Delta Tm \) is the average of the freezing point lowerings of the sample solutions and K is the water which delay, no signal was present for dry samples or ice at \(-2\) C. The uncorrected free induction decay signal was assumed proportional to the liquid water content after the Boltzmann temperature correction was approximated by multiplying all NMR signals by the absolute temperature.

The lines were fitted in the process of liquid water content determination. The lines fitted are the best fitting hyperbolae in the L_T versus T plots and the best fitting straight line in the L_T versus 1/T plots for the results between \(-2.5\) and \(-40\) C. Hardy Frontier rye was killed at \(-24 \pm 1\) C and tender was killed at \(-5 \pm 1\) C.

The crowns of plants for analysis were excised at the basal end and were 5 mm in length. Three segments of crown tissue fitted in a NMR tube were used for each analysis. The samples were cooled to \(-2.6\) C and freezing was initiated with ice crystals from a glass rod dipped in liquid nitrogen. Samples were cooled at 1 C intervals and held at this temperature until freezing reached equilibrium as determined from the quantity of liquid water. This process sometimes took as long as 90 min. Liquid water content as a function of temperature was determined from 0 to 40 C with a Bruker Mini Spec p20 spectrometer as described by Burke et al. (2). The spectrometer was equipped with a programmable temperature controller. Briefly, the method involved using a series of 90° pulses separated by 5 sec. The free induction decay after the second and each succeeding pulse was monitored 23 usec after the pulse center. After this 23 usec

**Fig. 1.** Freezing curves for hardy (□) and tender (○) Frontier rye. The liquid water content, L_T, is expressed in grams of liquid water per gram of dry sample. L_T is plotted versus temperature and the reciprocal of temperature. The lines drawn are the best fitting hyperbolae in the L_T versus T plots and the best fitting straight line in the L_T versus 1/T plots for the results between \(-2.5\) and \(-40\) C. Hardy Frontier rye was killed at \(-24 \pm 1\) C and tender was killed at \(-5 \pm 1\) C.

**Fig. 2.** Freezing curves for hardy (□), intermediately hardy (△), and tender (○) Kharkov winter wheat. See Figure 1 for a description of the plots. The hardy, intermediately hardy, and tender Kharkov wheat was killed at \(-18 \pm 1\), \(-9 \pm 1\), and \(-3 \pm 1\) C, respectively.

**Fig. 3.** Freezing curves for hardy (□) and tender (○) Cappelle-Desprez winter wheat. See Figure 1 for a description of the plots. Hardy Cappelle-Desprez wheat was killed at \(-11 \pm 1\) C and tender was killed at \(-2 \pm 0.5\) C.

**Fig. 4.** Freezing curves for hardy (□) and tender (○) Manitou spring wheat. See Figure 1, for a description of the plots. Hardy Manitou wheat was killed at \(-5.5 \pm 0.5\) C and tender was killed at \(-2 \pm 0.5\) C.
Table I. Calculated and Measured Freezing Data

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Killing Temperature</th>
<th>H₂O Content (%)</th>
<th>Sample Correlation Coefficient</th>
<th>ΔTm (°C)¹</th>
<th>Unfrozen H₂O at -40 °C (L/40)</th>
<th>Frozen H₂O at Killing Temperature %</th>
<th>% total H₂O</th>
<th>% freezable H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manitou (tender)</td>
<td>-2 (±0.5)</td>
<td>5.63</td>
<td>0.993</td>
<td>-1.65 (±0.07)</td>
<td>0.13 (±0.05)</td>
<td>0.37</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Cappelle Desprez (tender)</td>
<td>-2 (±0.5)</td>
<td>5.64</td>
<td>0.980</td>
<td>-1.39 (±0.12)</td>
<td>0.17 (±0.09)</td>
<td>0.33</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>Kharkov (tender)</td>
<td>-3 (±1)</td>
<td>5.27</td>
<td>0.999</td>
<td>-1.18 (±0.01)</td>
<td>0.10 (±0.01)</td>
<td>0.26</td>
<td>59</td>
<td>60</td>
</tr>
<tr>
<td>Frontier (tender)</td>
<td>-5 (±1)</td>
<td>5.07</td>
<td>0.999</td>
<td>-1.92 (±0.03)</td>
<td>0.18 (±0.02)</td>
<td>0.4</td>
<td>60</td>
<td>62</td>
</tr>
<tr>
<td>Manitou (hardy)</td>
<td>-5.5 (±0.5)</td>
<td>3.82</td>
<td>0.992</td>
<td>-1.71 (±0.08)</td>
<td>0.26 (±0.04)</td>
<td>0.34</td>
<td>63</td>
<td>67</td>
</tr>
<tr>
<td>Kharkov (semi-hardy)</td>
<td>-9 (±1)</td>
<td>4.15</td>
<td>0.997</td>
<td>-1.57 (±0.04)</td>
<td>0.18 (±0.02)</td>
<td>0.29</td>
<td>74</td>
<td>78</td>
</tr>
<tr>
<td>Cappelle Desprez (hardy)</td>
<td>-11 (±1)</td>
<td>2.85</td>
<td>0.979</td>
<td>-2.00 (±0.13)</td>
<td>0.22 (±0.04)</td>
<td>0.23</td>
<td>74</td>
<td>80</td>
</tr>
<tr>
<td>Kharkov (hardy)</td>
<td>-18 (±1)</td>
<td>2.73</td>
<td>0.991</td>
<td>-2.30 (±0.09)</td>
<td>0.24 (±0.03)</td>
<td>0.35</td>
<td>77</td>
<td>85</td>
</tr>
<tr>
<td>Frontier (hardy)</td>
<td>-24 (±1)</td>
<td>2</td>
<td>0.982</td>
<td>-2.94 (±0.17)</td>
<td>0.21 (±0.04)</td>
<td>0.26</td>
<td>79</td>
<td>88</td>
</tr>
</tbody>
</table>

¹ In grams liquid per gram dry sample.
² Sample correlation coefficient for a linear regression analysis between -2.5 and -40 °C.
³ Standard error in parentheses.

cannot be frozen to form crystalline ice. Values for these two parameters are given in Table I along with the liquid water present at the killing temperature.

Regardless of hardness levels, it is apparent that these samples exhibit a simple freezing behavior not unlike a dilute aqueous solution. The amount of water unfrozen at -40 °C ranges from 0.23 to 0.40 g/g dry sample (Table I). Similar amounts of liquid water have been reported for frozen protein and polypeptide solutions at -35 °C (6-8). The value of the parameter K in equation 1 provides a better measure of the unfreezable water. Neither K nor the amount of unfrozen water at -40 °C are correlated to the hardness level (Table I). For each of the varieties tested, the value of K increased slightly during cold acclimation. These results are in sharp disagreement with those of Macdowell and Buchanan (11) who reported almost no unfreezeable water in tender wheat samples. It would appear from these results that there is no simple relation between the hardness level and the nonfreezable or "bound" water contents of hardy and tender samples.

The freezing curves and thawing curves were identical if care was taken to avoid supercooling during the initial freezing process. Thus it does not appear that the water in these cereals exhibits metastable states during slow freezing as is the case in some woody plant species (2, 3). Also, there was no difference between the freezing curves obtained for living or dead samples irrespective of their hardness. No measurable amount of liquid water is associated with the living state as suggested by several hypotheses (13, 14).

As noted already by Metcalf et al. (12), the hardy cereals have significantly less water on a dry weight basis (Table I). These hardy samples, even with less total water present, survived a much higher proportion of that water frozen (Table I). For example, hardy Frontier rye with 2 g water/g dry sample tolerated 88% of its freezable water frozen before injury occurred, whereas tender Manitou spring wheat with 5.6 g dry sample was killed when only 18% of its freezable water was frozen. The most apparent difference between the crown tissues of cultivars differing in hardness is the ability of the harder crowns to tolerate diminishing quantities of liquid water. Also the parameter ΔTm, which is the freezing point lowering, tended to be larger for hardy samples (Table I).

Johannsson (4) and Johannsson and Krull (5) demonstrated that winter wheat leaves killed below -6 °C were killed when approximately 85% of their freezable water froze. This is in general agreement with the results reported here for crown tissue when the killing point was below -6 °C. The tender crown tissue killed above -6 °C tolerated much less freezing than the 85% Johannsson reports. The discrepancy between the two sets of data probably originates from disagreement in the killing temperatures reported for tender samples. We are confident that the viability test used here and the killing temperatures reported accurately reflect the hardness of the crown tissues. Johannsson (4) and Johannsson and Krull (5) studied freezing in leaf tissue and measured whole plant regrowth. Their results, which suggest hardness is acquired slowly by increasing the cell sap concentrations and reducing the proportion of water frozen at subfreezing temperatures, are not consistent with these results for crown tissue. The most noticeable change in crown tissue as it acclimates to cold is its increased tolerance of freezing. Crown tissue was studied because it is the important tissue for whole plant survival. Leaf or root tissue, if injured, can be regenerated if the-crown is healthy.

LITERATURE CITED