Freezing Injury in Potato Leaves

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ABSTRACT

Time-temperature profiles of freezing leaves from frost-resistant (Solanum acaule Bitt.) and frost-susceptible (Solanum tuberosum L. subsp. tuberosum Hawkes) types of potatoes did not reveal any major differences. The pattern of change in resistance of leaves to low voltage, low frequency current during freezing was different in the frost-resistant and susceptible leaves. In tissue sections from both types of leaves, cells freeze extracellularly at cooling velocities lower than 5 C per minute. Cells from leaves of resistant plants showed a higher osmotic pressure but not a higher water permeability than those from susceptible plants. The extent of injury caused by even very slow freezing was greater than that caused by equivalent isopiestic desiccation, particularly in susceptible leaves. The higher osmotic pressure in cells of leaves from resistant plants can account for the greater desiccation resistance but not for the frost resistance observed.

The yield of many crops, such as potato, is seriously reduced by partial and total frost damage to foliage during the growing season. Efforts to develop techniques for selecting resistant genotypes or reducing injury by cultural practices have been seriously hampered by our limited understanding of the critical lethal stress which occurs during freezing.

When plant tissues are subjected to subfreezing temperatures, ice initially forms in the extracellular spaces where the water contains less dissolved substances and freezes at a higher temperature. Crystallization often does not proceed into the protoplasts because the plasma membrane acts as a barrier to nucleation (4, 9). At relatively low rates of cooling the protoplasts initially supercool, but soon equilibrate by losing water to the extracellular ice, which has a lower vapor pressure, and thus avoid freezing. At rapid rates of cooling the protoplasts may become substantially supercooled as the plasma membrane restricts the movement of cellular water to extracellular ice. When the system is sufficiently displaced from equilibrium, the supercooled protoplasts may become nucleated either spontaneously or through the membrane resulting in intracellular freezing (4, 6). Intracellular freezing is almost lethal to plant and animal cells. Although extracellular freezing kills many tender plant species, numerous hardy perennial plants survive without injury at least at certain times of the year when they are frost hardened.

According to Mazur (6), the critical cooling rate above which intracellular freezing occurs depends mainly on the water permeability of the plasma membrane, the critical velocity being lower for cells with low permeability. The rate of cooling during natural frosts is low (usually a few degrees per hour) and intracellular freezing is believed to occur very rarely if at all in nature (4, 5).

During extracellular freezing the protoplasts become desiccated, because at a given temperature the aqueous vapor pressure over ice is less than that over supercooled water, and therefore water moves from the protoplasts to the extracellular ice. Injury from extracellular freezing is generally believed to be caused by the desiccation which results (4–6). Olien (19), however, believes that extracellular ice can cause mechanical damage when crystallization is rapid. He suggests that even when crystallization proceeds slowly and the amount of ice formed is a continuous function of temperature, factors other than simple desiccation may be involved in injury (10). Sufficient work to test these hypotheses has not been done, especially in frost-susceptible plants such as potato.

MATERIALS AND METHODS

Solanum acaule Bitt. (PI 210029) and S. tuberosum L. subsp. tuberosum Hawkes (var. Red Pontiac) were used in these studies. S. acaule is one of the hardest of the tuber-bearing Solanum species while Red Pontiac, like most cultivated varieties, has little frost resistance. Genotypically uniform plants were grown from rooted stem cuttings. After 4 weeks in the greenhouse they were transferred to a growth chamber and maintained for two weeks at a 12-hr photoperiod and a day/night temperature regime of 12 C/2 C, prior to study. The fifth through the eight leaf from the apex were used in all studies to reduce any age-dependent variability. Temperatures were monitored with cooper-constantan thermocouples and a multipoint recording potentiometer. Analysis of variance and f tests were used for assessing validity of results.

Freezing Curve Studies. Excised leaves with thermocouples attached were cooled in 15 cm long glass tubes placed in a cooling bath. The leaves were inoculated with ice crystals by touching them with a frost-covered pipe cleaner introduced into the tubes when the temperature reached −2 C. The temperature was subsequently lowered at the rate of 1°C per hr, while the leaf temperatures were recorded. Leaves were removed from the bath at different test temperatures and transferred to a refrigerator maintained at 0 C for slow thawing. After 2 hr in the refrigerator they were transferred to a humid chamber at room temperature and injury was estimated visually the next day.

Microscopic Observations. Leaf sections were frozen at different rates of cooling on a microscope stage equipped with a thermoelectric cooler. The 60- to 100-μm cross sections cut with a Vibratome were suspended in FC-47 fluorocarbon fluid:

1 Scientific journal series paper No. 7701 of the Minnesota Agricultural Experiment Station. This research was supported by a grant from the Rockefeller Foundation.

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(Minnesota Mining and Manufacturing, St. Paul, Minn.) during freezing. Sections were inoculated with a cold needle. Thawed sections were transferred to mannitol solutions and plasmolyzed twice. Ability to undergo plasmolysis and deplasmolysis was taken as an indication of cell survival.

**Electrophoretic Measurements.** An electrophoretic method developed by Olien (7) was used to study the patterns of freezing in leaves from resistant and susceptible genotypes. The resistance of tissues to low frequency or direct, low voltage applied current is a function of the relative amount of liquid water in films along the extracellular spaces, when the tissue is not injured (7, 8). Leaf strips 1 cm wide were placed between aluminum cooling blocks for freezing. Platinum wire and charcoal paste contacts were attached to the two ends of the sample and direct current at about 1 V per cm of leaf length was applied. The direction of current flow was reversed every half minute to prevent excessive polarization, and current was continuously recorded during freezing. The relative electrophoretic mobility \( M_i \) at temperature \( t \) was computed as follows:

\[
M_i = \frac{C_i}{C_s} \times \frac{V_s}{V_i}
\]

\( C_i \) and \( C_s \) are current passing through the sample at \( t \) and 0 C, \( V_s \) and \( V_i \) are values of the viscosity of liquid water at \( t \) and 0 C, respectively. According to Olien (7) \( M_i \) is equal to the proportion of unfrozen extracellular water at temperature \( t \), as long as there has been no injury to the tissue. \( M \) was evaluated at different subzero temperatures.

**Osmotic Pressure and Permeability Measurements.** Osmotic pressure was determined by the plasmometric method (11) which involves measurement of the volumes of plasmolyzed cylindrical protoplasts in hypertonic solutions. Water permeability constants were measured by following the time course of protoplast expansion during deplasmolysis via time-lapse photomicrography. Details of the method including formulas for computing permeability constants have been described by Stadelmann (11).

**Slow Freezing and Desiccation Studies.** Comparisons were made between the injury resulting from very slow freezing of intact leaves and from equivalent isopiestic desiccation of the leaves over sucrose solutions of different concentrations. In the slow freezing experiments leaves from both genotypes were placed on ice in Erlenmeyer flasks at 0 C in a modified (12) domestic freezer. Fluctuations from the mean temperature inside the flasks were less than 0.1 C. The temperature was maintained at 0 C overnight. During the next day the temperature was gradually lowered to \(-1\) C and samples were then held at this temperature overnight. The temperature was lowered 1 C per day in this fashion until the desired test temperature was reached. The leaves were kept in continuous contact with ice to avoid supercooling, and the slow cooling rate was used to avoid intracellular freezing and other injurious forms of nonequilibrium freezing. Samples were thawed at 0.5 C per hr before estimating injury by electrolyte leaching.

The desiccation energy, or free energy difference \( \Delta F \) of ice with respect to pure water can be calculated thus:

\[
\Delta F = RT \ln \frac{P_i}{P_s}
\]

where \( P_i \) is the vapor pressure over pure water at \( T \) degrees absolute, \( P_s \) the vapor pressure over ice at the same temperature, and \( R \) the gas constant. In order to use this formula for frozen plant tissue, one must assume that the potential energy of association of water with substances in the cell is not affected by the proximity of ice (10).

The same formula can be used to calculate the desiccation energy, with respect to pure water, of the atmosphere over solutions with different vapor pressures. \( P \) is then the aqueous vapor pressure over the solution. Using the values experimentally determined by Berkeley et al. (1) for vapor pressure over sucrose solutions at 0 C, sucrose solutions of different concentrations were made up. Concentrations were used which had the same desiccation energies at 0 C as ice would have at the various test temperatures studied. Thus, protoplasts of leaf samples at vapor pressure equilibrium over these solutions were subjected to the same desiccation stress as the samples which were slowly frozen to the corresponding subzero temperatures. Unfrozen leaf samples from both species were desiccated over these solutions in sealed containers at 0 C in the freezer described above. By monitoring the weight of samples in preliminary runs, it was found that it took about 10 days for the samples to reach constant weight. In the final runs the leaves were desiccated for 15 days. Leaves were rehydrated in a water-saturated atmosphere overnight at room temperature and injury was estimated by the leaching technique.

**Leaching Technique for Evaluating Injury.** Leaves were washed in distilled water before the experiments. After subjection of freezing or desiccation stress individual leaf samples were placed in 15 ml of double-distilled deionized water and shaken in a water bath at 25 C for 1 hr. The extract was decanted, and its conductance \( (C_1) \) was measured at 400 cycles. The samples were then killed by immersion in liquid nitrogen for 5 min, placed back in the original extract, and shaken once more for 1 hr at 25 C. The final conductance \( (C_2) \) was then measured. \( L = C_1/C_2 \) was used as index of injury. The technique (2) is based on the fact that plasma membranes lose their semipermeability when the cells are injured. Injured tissues leach proportionately greater amount of electrolytes into the surrounding water. Values of \( L \) higher than 0.5 are an indication of severe injury.

**RESULTS AND DISCUSSION**

**Freezing Curves.** Freezing curves of the leaves from the resistant \( (S. acaule) \) and the susceptible \( (S. tuberosum) \) genotypes had the same general appearance. The freezing plateau of the single exotherm observed occurred between \(-1\) and \(-2\) C in both cases. The killing points, however, were \(-2.5\) C for \( S. tuberosum \) and \(-5.5\) C for \( S. acaule \). Figure 1 shows a generalized freezing curve on which the killing points are indicated. It appears that leaves of both types can withstand some ice formation in their tissues. \( S. acaule \), however, can tolerate a greater amount of ice formation than the susceptible species, since it is killed at a lower temperature. It is known (6, 9) that the lower the temperature, the greater the amount of ice double exotherm in the thermal differential profiles of freezing formed in freezing tissues. Hudson and Idle (3) observed a \( S. acaule \) leaves when the thermodisjunction was inserted in the petiole. In this study thermojunctions were affixed to the leaf surface.

**Microscopic Observations.** Microscopic sections of leaves of both genotypes froze extracellularly at cooling rates of 4 C per min or slower. Cells, including cell walls, contracted visibly as the temperature was lowered. Although tissue sections were not as frost resistant as intact leaves, there were detectable differences between the two genotypes. Most \( S. acaule \) cells would plasmolyze after freezing to \(-3\) C, but very few \( S. tuberosum \) cells survived. When the sections were incubated at \(-2\) C and the temperature was then lowered to \(-12\) C in 2 min, intracellular freezing could be observed as darkening or "flash-
ing" of individual cells. The frequency of occurrence of flash-
ing was approximately the same in both types of tissue. Similar results were obtained when tissue sections were inoculated after supercooling to −10°C.

Levitt (5) has suggested that cells of freezing tissue sections lose water at a faster rate than cells in intact plant organs sub-

Table I. Osmotic Pressure and Water Permeability of Palisade Cells from Frost-resistant S. acaule and Frost-susceptible S. tuberosum Leaves

Osmotic pressure was computed from cell volume data of 24 cells of each species. Six cells were measured in each of four different hypertonic solutions. The water permeability constant for each species was computed from time-volume curves of six deplasmolyzing cells from two different sections.

<table>
<thead>
<tr>
<th>Species</th>
<th>Osmotic Pressure ± SE</th>
<th>Water Permeability Constant K ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. tuberosum</td>
<td>7.4 ± 0.40</td>
<td>28.7 ± 3.26</td>
</tr>
<tr>
<td>S. acaule</td>
<td>10.4 ± 0.34</td>
<td>21.5 ± 0.79</td>
</tr>
</tbody>
</table>

Table II. Injury to Resistant (S. acaule) and Susceptible (S. tuberosum) Potato Leaves after Freezing and Desiccation

L values (leaching ratios) are used to express injury. Values above 0.5 indicate that leaves were killed or severely injured. Each value is the mean of four observations.

<table>
<thead>
<tr>
<th>Freezing Temperature</th>
<th>Injury from Freezing</th>
<th>Sucrose Solution Used for Desiccation</th>
<th>Injury from Desiccation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
<td>Susceptible</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−2</td>
<td>0.13 ± 0.018</td>
<td>0.13 ± 0.014</td>
<td>0.77</td>
</tr>
<tr>
<td>−3</td>
<td>0.14 ± 0.032</td>
<td>0.70 ± 0.064</td>
<td>1.07</td>
</tr>
<tr>
<td>−4</td>
<td>0.20 ± 0.031</td>
<td>0.90 ± 0.019</td>
<td>1.28</td>
</tr>
<tr>
<td>−5</td>
<td>0.38 ± 0.064</td>
<td>0.89 ± 0.026</td>
<td>1.47</td>
</tr>
<tr>
<td>−6</td>
<td>0.65 ± 0.078</td>
<td>0.91 ± 0.018</td>
<td>1.62</td>
</tr>
<tr>
<td>−7</td>
<td>0.84 ± 0.047</td>
<td>0.93 ± 0.015</td>
<td>1.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>0.11 ± 0.023</td>
<td>0.11 ± 0.023</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.15 ± 0.010</td>
<td>0.14 ± 0.011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.21 ± 0.017</td>
<td>0.25 ± 0.064</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25 ± 0.059</td>
<td>0.55 ± 0.025</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.45 ± 0.053</td>
<td>0.76 ± 0.060</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.64 ± 0.084</td>
<td>0.90 ± 0.036</td>
<td></td>
</tr>
</tbody>
</table>

1 L ± SE.
jected to freezing; consequently the critical cooling velocity would be higher in the former. But since 5 C per min is more than 60 times the normal rates of cooling under natural conditions, it seems likely that extracellular freezing is the normal occurrence during natural frosts unless initiation of freezing is preceded by substantial supercooling.

**Electrophoretic Measurements.** Results of electrophoretic mobility studies are shown in Figure 2. The apparent relative mobility increased in the susceptible leaves soon after freezing and then decreased. This may indicate the early occurrence of injury in susceptible leaves. After injury the plasma membranes would cease to be ionic barriers and therefore, the electrical resistance of the tissue would decrease.

In hardy leaves mobility decreased smoothly, indicating that there was a continuous exponential decrease in the amount of unfrozen extracellular water as the temperature decreased. In the mobility profile, as in the freezing curve, there was no indication of double freezing. Freezing apparently proceeded at equilibrium. Similar mobility patterns have been found in cereals (8).

**Osmotic Pressure and Water Permeability Measurements.** The osmotic pressure of cells of the two genotypes was significantly different but the water permeability constants were not (5% significance level). High osmotic pressure and high water permeability have often been found in frost hardy cells (4). However, the absence of a substantial difference in water permeability between the cells of the two genotypes is consistent with the fact that intracellular freezing occurred with equal ease during rapid cooling in both genotypes (Table I).

**Slow Freezing and Desiccation Studies.** Results are given in Table II, and mean values of the leaching ratio (L) are plotted against desiccation energy in Figure 3. In both resistant and susceptible leaves, injury from freezing to —6 C was significantly higher (5% level) than injury from equivalent desiccation. Freezing caused more injury than desiccation at corresponding levels of desiccation energy, especially in the susceptible genotype. This indicates that factors other than simple desiccation are involved in slow freezing injury.

Similar results were obtained by Olien (10) in recent work on barley in which the desiccation energy was varied independent of the temperature in frozen samples, by creating temperature differentials in the sample freezing chambers. Olien suggests that frost desiccation should be considered as one stress vector in freezing and that other effects of low temperature, and changes caused by the proximity of ice, may also be important.

Figure 3 shows that the frost resistant *S. acaule* had more resistance to drying than *S. tuberosum*. This difference can be explained on the basis of the higher osmotic pressure of *S. acaule* cells. The desiccation energy corresponding to L = 0.5, during desiccation stress, was 32 cal/mole for *S. acaule* and 25 for *S. tuberosum*. These desiccation energies correspond to sucrose solutions having osmotic pressures of 76 and 58 respectively at 0 C, values which are 7.3 and 7.9 times the measured osmotic pressures of the *S. acaule* and *S. tuberosum* cells. Thus, the desiccation stress causing death in the two genotypes was essentially the same. But this difference in osmotic pressure is insufficient to account for the difference in frost resistance between the two genotypes.

In summary, the results indicate that the frost resistance exhibited by *S. acaule* is true frost tolerance, i.e., ability to withstand extracellular ice formation to —5 C; that this resistance is not related to a relatively greater capacity for avoiding intracellular freezing during rapid cooling; and that injury to potato foliage from extracellular freezing probaby cannot be explained in terms of simple frost desiccation.

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**LITERATURE CITED**