The relation of bound water to winter hardiness in the apple

Arvil L. Stark
Iowa State University

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THE RELATION OF BOUND WATER TO WINTER HARDINESS IN THE APPLE

by

Arvil L. Stark

A Thesis submitted to the Graduate Faculty for the Degree of
MASTER OF SCIENCE

Iowa State College
1931
ACKNOWLEDGMENTS

Acknowledgment is made to Professors B. S. Pickett, T. J. Maney and W. E. Loomis for their direction and suggestions in carrying out this investigation. We are indebted to the Dairy and Botany Departments for the use of freezing equipment and the hydraulic press.
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INTRODUCTION

Up to the present time no satisfactory test has been devised to separate hardy from non-hardy plants. Such a test would add a great deal to the rate of progress in fruit breeding work in rigorous climates.

The determination of hardness of hybrids by "test winters" naturally involves a period of years. A measure, in the first or second year, of the tree's resistance to cold, would make possible the elimination of the more tender hybrids at the start. Where thousands of seedlings are produced each year this elimination would be of great importance.

A theory prevails among those who have studied the effects of cold on plants that hardness is a water relationship. It is believed that death from freezing is due to desiccation, ice forming within the tissue at the expense of the water in the protoplasm. If the ice formation proceeds beyond critical limits, the protoplasm dies from the water loss. Thus, plant tissues holding greater quantities of water against freezing should be more hardy than those with less capacity for water retention, and a measure of the quantity of water not frozen, when a tissue is subjected to low temperatures, would be an indication of the cold resistance of the plant.

On the basis of this assumption the following investigation was made in an effort to discover the relation between the
quantity of water held against freezing, and expression under pressure, and the winter hardiness of apple varieties.
The literature on the subject of hardiness is so extensive that a complete review of the writings dealing with the general problem is not included here, since excellent reviews are to be found in publications cited as follows: (1), (3), (13), (22), (23), (48), (51), (57) and (66). Only those publications more closely related to the bound water phase of the hardiness problem are briefly reviewed.

Bouyoucos (6) worked with seeds of grains, clover, peas and beans which had been soaked in water for two days. By use of the dilatometer he found that they caused 25.05 to 76.76 percent of their water content to become unfree. He attributes their power to absorb water partly, if not largely, to their tremendous internal osmotic pressure caused by a high content of easily water-soluble material.

With experiments on lettuce and beets grown in soil where nutritive substances were added, Crist (10) found that the plants under different treatments varied greatly in their water content and retention ability. He applied nutritive substances to deficient soils and produced plants with increased total water content per unit dry weight and an inhibition of water loss against evaporation. By adding nutritive substances to the soil he was able to increase the amounts of bound water in spinach and lettuce. This increase he attrib-
utes to the raising of the hydration capacity or to increasing
the relative quantity of cellular substances which are hy-
dratable.

On the other hand, McCool and Millar (37) using rye,
wheat, corn, sweet and red clover found no increase in the
amount of water that was retained unfrozen at -2.5 and -4.0°C.
when the density of the soil solution in which the different
plants were grown was increased.

According to Dorsey and Strausbaugh (13), plum varieties
have a critical temperature, a point where a sufficient amount
of water is withdrawn to cause permanent injury to the proto-
plasm. "If the imbibitional forces within the protoplasm are
strong enough to retain the water against the dehydrating
effect of freezing, the critical temperature is much lower
and the variety is classed as hardy."

As measured by the dilatometer method, Lett (29) found a
direct correlation between hardiness, as observed from ex-
perience, and the percentage of bound water in the cortex of
bramble canes.

In his work with wheat plants Martin (33) concludes that
the most important character influencing hardiness is the
ability to build up a high imbibition pressure of the cell
colloids during hardening. He states that although Swedish
rye, which is more hardy than any wheat, has a high moisture
content, a low percentage of total solids and a low freezing-
point depression like non-hardy wheats, still it has a lower rate of respiration at low temperatures and a greater imbibition pressure than the hardiest wheats.

Pitch pine leaves, according to Meyer (40), form a physico-chemical organization at the approach of winter that resists the formation of ice in the leaf tissues. He found that the percentage of bound water, as determined by the volume of the expressed liquid, increased at the expense of the free water during the winter months.

Newton (44) concluded from his experiments on six varieties of wheat that the volume of the press-juice expressed per 100 grams of hardened leaves was inversely proportional to the hardiness of the variety, while in unhardened leaves no such relation could be found. The quantity of hydrophilic colloids in the press-juice was directly proportional to hardiness.

In his work with insects, Robinson (52) showed by the heat-of-fusion method that a tender species, when subjected to low temperatures over a period of time, was unable to increase its percentage of bound water as contrasted with a hardy species. A species of intermediate hardiness was also intermediate in the percentage of bound water it contained when hardened. He attributes the water binding chiefly to colloidal properties in the protoplasm, since a depression of the freezing-point did not always accompany hardiness.

Hardened cabbage leaves frozen at -3° and -4°C. yielded
only two-thirds as much water to freezing as like plants not hardened. Thus Rosa (57) found more water remaining unfrozen, as determined by the dilatometer method, in hardened than in tender leaves, although the moisture content of the hardened plants was less.

Weimer (63), using the dilatometer, was able to correlate the amount of bound water and the state of hardening within a variety of alfalfas, but no such correlation was found to exist in variety comparisons.
MATERIAL

Material used in this investigation consisted of shoots of one season’s growth taken from vigorous 4 to 5 year old nursery stock grown especially for the purpose of this study.

Samples were collected in the morning of the day they were to be tested. These were immediately taken into the laboratory and stripped of their leaves. Shoots of a variety were then cut into pieces about 1½ inches in length. These pieces were thoroughly mixed and 20 gram samples were quickly weighed on a torsion balance. From the balance they were emptied into large test tubes which were immediately stoppered. This tissue was used in the heat-of-fusion method and for determining specific heat.

Other samples of ten grams each were weighed and dropped into air-tight bottles containing sufficient ligroin to cover the tissue. These samples were used when making determinations with the dilatometer.
METHODS OF PROCEDURE

At the beginning of this study freezings were made by immersing the samples contained in the test tubes and dili-
tometers in the circulating brine of a cold storage plant. It was impossible to use this machine during the apple storage season, however, so an insulated metal box that held about 55 liters of cooling brine was constructed. Zinc containers of about 10 liters capacity were filled with salt and ice to cool the brine. Two of the zinc boxes were submerged in the brine solution which was circulated by an electric stirring device. By refilling with salt and ice it was possible to maintain a temperature as low as \(-15^\circ\text{C}\).

Because it was impossible to reach the desired low tem-
peratures with the above apparatus, a mechanically refrigerated ice cream cabinet was finally employed. With this machine a constant temperature was obtained when the freezing-point of the cooling brine was maintained at the desired freezing tem-
perature. The brine was circulated by an electric stirring device to keep the temperature uniform throughout the container.

The test tubes containing the samples were held in the cooling brine by means of a perforated wooden lid which was tightly fitted to the top of the cooling chamber. Samples were placed directly into the cold brine without any previous cooling.
Heat-of-fusion Method

The heat-of-fusion method is based upon the fact that ice absorbs heat when changing to water. By measuring the amount of heat used in this change of phase it is possible to calculate the quantity of ice involved. Thoenes (62) and Robinson (63) have used this method to measure bound water in animal tissue.

The procedure followed is a form of calorimetry modified to the use of biological material. In brief, it consists of measuring the heat absorbed by the ice when changing to water in the frozen tissue. This heat is measured by dropping the frozen tissue into a definite quantity of water in which the temperature difference can be accurately determined.

In this study an ordinary wide-mouth thermos bottle of one pint capacity was used for a calorimeter. A rubber stopper, in which were fixed a thermometer and stirrer, was fitted in the mouth of the bottle. The thermometer, 60 cm. in length and graduated to tenth degrees, reached within 2½ cm. of the bottom of the bottle when the stopper was in place. This and the other thermometers used in the investigation were checked with a U. S. Bureau of Standards instrument.

Stirring, which is necessary for accurate temperature readings in the bottle, was accomplished by the vertical movement of a stirring rod, to the end of which was attached a circular piece of copper screen slightly smaller in diameter than
the inside of the bottle.

When making a determination, 200 ml. of water were pipetted into the bottle. The temperature of the frozen sample was next recorded, after which the temperature in the calorimeter was read while the water was being stirred. The frozen sample was now removed from the cooling brine and emptied carefully into the calorimeter so as to prevent splashing of water on the walls of the bottle. This transfer was made as quickly as possible to prevent any change in the temperature of the sample. Continuous stirring followed until the drop in temperature ceased when a second reading was made. The tissue was then taken from the calorimeter and put in weighing bottles for dry weight determinations.

The water lost from the tissue during freezing collected on the sides of the test tube. This water was weighed and added to the quantity of free water determined by calculation.

Robinson (55) found that the drop in temperature of the water in the calorimeter did not give full value of the heat-of-fusion because of the heat lost from the stirrer, thermometer and walls of the bottle. Correction for this error can be made with pure ice. The procedure was as follows: Ten gram samples of boiled distilled water were accurately weighed in small glass vials which were immediately stoppered. These were cooled in test tubes containing calcium chloride to prevent moisture accumulation on the outside of the vials.
After freezing for several hours, vial and ice were emptied into the calorimeter from the freezing test tube. The resulting temperature change in the bottle would include that due to the vial as well as the ice. After the calories used to warm the vial were calculated its influence was eliminated by subtraction. Then on the basis of 80 calories per gram of ice it was determined how much additional heat was absorbed other than from the water. Dividing the number of calories actually taken up by the ice, by the number of calories change in the water, a factor is obtained which compensates for the heat lost from the apparatus. Several determinations were made in order to verify results.

The factor, 1.059 in this case, is only applicable under definite conditions of water level and temperature change in the bottle. It was found that 10 grams of ice gave the approximate temperature change of 20 grams of tissue if the water level was raised to bring it to the same height as when the tissue was tested. If the factor obtained is multiplied by the number of calories change in the water in the bottle, the correct value for heat-of-fusion is obtained.

Another correction is necessary if the mid-point of the temperature change in the bottle is not the same as room temperature. Variations in this respect were never greater than $1.5^\circ$C. and generally within $1.0^\circ$, making a correction so small that it was omitted.
In calculating the amount of frozen water by this method it is necessary to know the specific heat of the material tested. To determine this value the procedure described in the heat-of-fusion method was followed, with the exception that the tissue was cooled only to 0°C. or slightly above to make sure that no ice formation took place in the sample. Specific heat was then calculated by the usual method employed in physics.

It is also necessary to know the freezing-point of the material tested. An explanation of the procedure followed to determine this temperature is included under the dilatometer.

Employing the data obtained in the operations outlined above, the frozen water within the tissue can be calculated by the following formula as given by Robinson (53):

\[ X = \frac{FM(T_3 - T_4) - SN(T_2 - T_4)}{80} = \frac{T_2 - T_1}{2} \]

In the formula:

- \( P \) = factor as calculated above
- \( N \) = number of ml. of water used in bottle
- \( T_3 \) = temperature of water at beginning of test
- \( T_4 \) = temperature of water at the end of test
- \( S \) = specific heat of similar material
- \( W \) = weight of material tested
- \( T_2 \) = temperature of material while frozen
- \( T_1 \) = freezing point of similar material
- \( 80 \) = calories to change 1 gram of ice to water

\( X \) = grams of free or frozen water within the tissue. Unfrozen or bound water is obtained by subtracting this figure from the total water content of the tissue.
The Dilatometer

As the name implies, the dilatometer measures the dilation or expansion of water when changing to ice. Rosa (57), McCool and Millar (37), Lott (29) and others have used the dilatometer for measuring frozen water in plant tissue. Bouyoucos (4) describes in detail a dilatometer of his own design for measuring different forms of water in soil. Two 50 ml. and two 75 ml. Bouyoucos type dilatometers were used in this study.

In brief, a Bouyoucos type dilatometer consists of a cylindrical bulb with an opening at one end for insertion of a thermometer. Readings of expansion are made on a graduated tube which is fused in the side of the bulb near the open end. This tube is parallel with the long axis of the bulb and graduated to 0.01 ml.

Determinations with the dilatometer are usually made as follows. Material to be tested is dropped into the bulb which is then completely filled with ligroin. After the thermometer is seated tightly in place the graduated tube is also filled. Then the bulb and its contents are under-cooled below the freezing-point of the material in the bulb. When a constant temperature is reached, as indicated by cessation of movement in the column of ligroin, the height of the column is recorded. Ice formation in the material is then caused by further cooling or jarring. After the formation of ice the bulb is brought to the temperature at which the first reading was made. At this
temperature the height of the ligroin column is again recorded. The difference between the first and the second readings in the height of the ligroin column is the expansion due to ice formation.

It is clear that by following this method of procedure it is possible to measure only the quantity of ice frozen at temperatures slightly below the freezing-point of the material. In order to compare the accuracy of the dilatometer method, which measures an expansion of 0.1 of a ml. per gram of water, with the heat-of-fusion method, which measures 80 calories per gram of water, the temperatures at which the duplicate samples are frozen should be the same.

In an attempt to use the dilatometer at the low temperature used in the heat-of-fusion method the procedure was as follows. Samples to be tested were dropped into the bulb of the dilatometer which was then filled with ligroin. After the tissue was stirred with a glass rod to remove air bubbles the thermometer was fitted in place and the graduated tube filled with ligroin. The bulb of the dilatometer was then submerged in a bath in which the temperature was slightly higher than the freezing-point of the tissue. When a constant temperature was reached in the bulb, the height of the ligroin column was recorded. The sample was then frozen along with samples to be tested with the heat-of-fusion method. At the end of the freezing period the dilatometer was transferred to a bath
held at a temperature of 0°C.

At this temperature rapid warming of the ligroin occurred, leaving the tissue frozen. Thus it was possible to bring the ligroin back to the temperature where the first reading was made while the tissue was still frozen. A second reading of the ligroin column was made when this temperature was reached. In this way the expansion due to ice formation at the lower temperature was determined.

At the conclusion of the first trial it was obvious that this method of procedure was impracticable because of the errors involved. While rapid warming was taking place in the ligroin there was a temperature difference between the outside and the center of the bulb. This difference caused differences in the expansion of the ligroin and thus altered the height in the tube. With the cold tissue in the bulb this error could not be accurately determined. It is also possible that some ice was melted during the rise in temperature, thereby making the expansion reading too small. A third error could be attributed to the inaccuracies in reading the thermometer and ligroin column while they were moving.

Another attempt to use the dilatometer in comparison with the heat-of-fusion was made as follows. The sample was put in the bulb and the ligroin added, after which the thermometer was set in place and the graduated tube filled as described above. After these preliminary steps the bulb and
its contents were cooled to 0°C., at which temperature the ligroin was brought up to the top graduation on the tube. The sample was then frozen along with duplicate samples to be tested by the heat-of-fusion method. At the end of the freezing period the height of the column was recorded while the dilatometer was still in the cooling brine. The change in height of ligroin as noted on the graduated tube was the result of both contraction in the ligroin and expansion due to ice formation in the tissue.

To find the amount of contraction in the ligroin, the number of ml. surrounding the tissue was multiplied by the contraction per ml. for the temperature change from 0°C to the freezing temperature. This contraction per ml. for the temperature change was found by making tests with ligroin alone in the dilatometers. The volume of liquid surrounding the tissue was found by subtracting the volume of the sample, as determined by water displacement in a burette, from the total volume of the dilatometer.

After the volume change in the ligroin was calculated, the expansion of the tissue could be obtained by subtraction. Most values found by this procedure showed a decrease rather than the expected increase in the volume of the tissue. From the results it appeared as if the contraction in the wood was, in most instances, greater than expansion from ice formation. In the few cases where readings indicated an expansion in tissue
the values were too small to account for the quantity of water frozen, as determined by the heat-of-fusion method. Because of the inconsistencies in the results obtained by this procedure it was not given further trial.

No further attempts were made in an effort to compare the dilatometer method with the heat-of-fusion method. It is pointed out in the descriptions above that the data are valueless for comparison with the heat-of-fusion method and for this reason none are recorded here.

Freezing-point Determinations

To find the freezing-point of a tissue samples were prepared as described in the dilatometer methods. After preliminary preparation the dilatometer was cooled in a bath at 0°C. From the bath at 0°C, the dilatometer was transferred to another bath in which the temperature was slightly lower than 0°C. If after a vigorous jarring no solidification took place at this temperature, the sample was transferred again into a colder bath. Successively colder temperatures were used until solidification was apparent, as indicated by a sudden rise in the ligroin column. The temperature at which solidification took place was recorded as the freezing-point of the tissue.

Although possibly not as accurate as other methods that might have been employed to determine freezing-points, this method gives an indication of the freezing-point of the tissue. From the data in Table V it can be seen that more water is
frozen at -20°C. than at -10°C. Under such conditions it seems possible that a single temperature would not include the freezing-points of all liquid frozen, since part of the ice solidified at one temperature and part at another.

Sap Expression Under Pressure

The ability of a tissue to retain water against physical pressure has been used to indicate the state of hardness in plants, hardier tissue holding a higher percentage of its water than similar tender tissue. In an effort to find whether or not this test could be used to separate hardy from tender apple varieties the following procedure was employed.

Shoots were cut into pieces about 1½ inches long and ground through a Wiley mill. The ground samples were kept in jars fitted with air-tight glass covers.

One set of samples was held 20 hours at a temperature of approximately -21°C., while another set was placed in cold storage at -1°C. The low temperature samples were allowed to thaw before pressing.

Pressings were made on a simple laboratory hydraulic press. The cross section area of the cylinder of the press was four square inches. Two felt pads on either end of the tissue served to reduce readsoption of expressed sap after the pressure was released.

Thirty grams of tissue were rapidly weighed and emptied into the cylinder of the press from the balance. Pressure in
the cylinder was brought to 5,000 pounds per square inch and immediately released. Piston and cylinder were then inverted to give better drainage of the sap. Again the pressure was brought to 5,000 pounds per square inch, where it was held for one minute.

Immediate weighing of the press-cake followed expression. The difference between the first weighing and the weight of the press-cake was regarded as the weight of expressed sap. Total water content of the tissue was found by drying the press-cakes.

After the tissue was killed by drying at 80°C, it was soaked with water and pressed in the same manner as the green tissue. The greater quantity of water held by the green tissue was attributed to the retention capacity of the living colloidal system.

Sap from the green samples was collected in weighing bottles for determination of percentage of dry matter.
EXPERIMENTAL RESULTS

At the beginning of the investigation it was intended that determinations should be made with the heat-of-fusion method at regular monthly intervals during the spring and summer. At the approach of maturity in the fall a two week interval was to be employed. By following this program a rather complete knowledge of the water retaining capacity of the shoots against freezing would be shown for an entire growing season, thereby giving some idea of the changing water relations with the approach of maturity in the fall.

Because of rearrangements and breakdown in the freezing apparatus the data are not as complete as desirable. In spite of this incompleteness the available data present some indication of the relationship of unfrozen water to maturity and hardiness.

Varieties chosen for comparison include a range of hardiness, as noted from horticultural experience, from extremely hardy to tender. Stayman, which is least hardy of the varieties used, was not available until late in the season and was not used in the comparisons in Tables I and II.

Samples used in the heat-of-fusion method varied from 20 grams by not more than 0.2 grams. They were weighed with an accuracy of 0.005 grams.
Unfrozen Water in Shoots Held at -10°C.

Table I shows the percentage of water and the percentage of water unfrozen in tissue tested at different periods of the year. The percentage of water is calculated on a green weight basis, while the percentage of water unfrozen is figured from the total water content of the tissue. In the table the varieties are arranged in order of their hardiness. Hibernal, the most hardy, is listed at the extreme left, and the others follow in order to the most tender in the last column on the right.
Table I

Unfrozen Water in Shoots Held at -10°C. for 4 Hours

(Determined by heat-of-fusion method)

<table>
<thead>
<tr>
<th>Date</th>
<th>Hibernal</th>
<th>Virginia</th>
<th>Wealthy</th>
<th>Jonathan</th>
<th>Delicious</th>
<th>Grimes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>water</td>
<td>unfrozen</td>
<td>water</td>
<td>unfrozen</td>
<td>water</td>
<td>unfrozen</td>
</tr>
<tr>
<td>May 17</td>
<td>58.4</td>
<td>43.3</td>
<td>58.6</td>
<td>42.5</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>June 17</td>
<td>70.6</td>
<td>22.6</td>
<td>67.7</td>
<td>25.4</td>
<td>69.2</td>
<td>23.0</td>
</tr>
<tr>
<td>July 18</td>
<td>65.1</td>
<td>30.6</td>
<td>61.3</td>
<td>35.2</td>
<td>61.0</td>
<td>34.1</td>
</tr>
<tr>
<td>Aug. 19</td>
<td>63.0</td>
<td>33.2</td>
<td>57.3</td>
<td>42.0</td>
<td>61.0</td>
<td>37.2</td>
</tr>
<tr>
<td>Sept. 24</td>
<td>52.4</td>
<td>46.0</td>
<td>53.1</td>
<td>47.2</td>
<td>51.3</td>
<td>50.2</td>
</tr>
<tr>
<td>Dec. 2</td>
<td>46.9</td>
<td>53.5</td>
<td>47.6</td>
<td>51.6</td>
<td>47.2</td>
<td>54.3</td>
</tr>
<tr>
<td>Jan. 9</td>
<td>44.4</td>
<td>58.1</td>
<td>45.8</td>
<td>60.5</td>
<td>46.2</td>
<td>53.9</td>
</tr>
<tr>
<td>Increase</td>
<td>150.0</td>
<td>138.2</td>
<td>134.4</td>
<td>128.4</td>
<td>125.2</td>
<td>104.0</td>
</tr>
<tr>
<td>Decrease</td>
<td>37.1</td>
<td>35.3</td>
<td>33.2</td>
<td>34.0</td>
<td>30.7</td>
<td>30.0</td>
</tr>
</tbody>
</table>
The increase in the percentage of unfrozen water from June to January is the most outstanding feature brought out in Table I. These percentage figures show the greatest increase in Hibernal, which is followed in order by consecutive smaller percentages for the more tender varieties, the lowest percentage increase of any variety is found in Grimes, the most tender.

There is some question as to the relative hardiness of Jonathan and Delicious in other localities, but at this station Jonathan has been observed to be somewhat more hardy. By placing Delicious below Jonathan in order of relative hardiness there appears to be a direct correlation between the percentage increase in unfrozen water from June to January and the winter hardiness of the varieties tested. Because the number of varieties worked with is so small, this correlation could have been accidental, but the large differences between the hardy and tender varieties seem to justify the assumption that such a correlation may exist. There is a difference of 46 percent between the most hardy and the most tender variety, or practically one-third of the total percentage increase in the most hardy.

It appears logical to make comparisons on the basis of percentage increase within a variety rather than on percentages remaining unfrozen at any one time, because different varieties may not require the same quantity of water to retain life in their protoplasm. Thus it would be the ability of a variety to
increase its water retaining capacity at the approach of winter that would indicate its hardness, rather than the percentage of water it could hold against freezing as compared with other varieties. When using this basis for comparison any differences in the water requirements of a variety would be eliminated. At least it is evident that the varieties cannot be arranged in order of their hardness by the percentage of water remaining unfrozen at any single determination. It is possible, however, that such an arrangement might have been found at some date between September 24 and December 2.

It is observed that along with the increase in the percentage of water not frozen there is also a decrease in total water content of the tissue. This decrease in total water would cause an increase in the percentage of water not frozen, even though the actual quantity remained unchanged. To eliminate the effect of this inter-relationship the amount of water not frozen was calculated on the basis of grams per gram green weight of tissue. The results of these calculations are recorded in Table II.
Table II

Grams Unfrozen Water Per Gram Green Weight of Tissue

<table>
<thead>
<tr>
<th></th>
<th>Hibernal</th>
<th>Virginia</th>
<th>Wealthy</th>
<th>Jonathan</th>
<th>Delicious</th>
<th>Grimes</th>
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<tr>
<td>May 17</td>
<td>.254</td>
<td>.249</td>
<td>.246</td>
<td>.254</td>
<td></td>
<td></td>
</tr>
<tr>
<td>June 17</td>
<td>.160</td>
<td>.171</td>
<td>.168</td>
<td>.172</td>
<td>.183</td>
<td></td>
</tr>
<tr>
<td>July 18</td>
<td>.199</td>
<td>.204</td>
<td>.208</td>
<td>.215</td>
<td>.241</td>
<td></td>
</tr>
<tr>
<td>Sept. 24</td>
<td>.251</td>
<td>.250</td>
<td>.260</td>
<td>.260</td>
<td>.223</td>
<td>.239</td>
</tr>
<tr>
<td>Dec. 2</td>
<td>.251</td>
<td>.245</td>
<td>.256</td>
<td>.251</td>
<td>.252</td>
<td>.237</td>
</tr>
<tr>
<td>Jan. 9</td>
<td>.258</td>
<td>.265</td>
<td>.249</td>
<td>.254</td>
<td>.269</td>
<td>.260</td>
</tr>
</tbody>
</table>

Percent increase: 61.3 | 55.0 | 56.5 | 51.2 | 56.4 | 42.1

It is evident from Table II that there is a difference in quantity of water held unfrozen in June and in January. The greater amounts unfrozen in January indicate that the decrease in total water content, as shown in Table I, is not alone responsible for the large percentage increases.

Just what part of this greater water retention capacity is due to accumulation of colloidal materials and what part is due to rise in osmotic value of the cell sap, it is difficult to determine. It is possible that both are influential, since the freezing-point was lowered and the dry content of the shoots increased as the season advanced toward maturity. The increase in dry matter indicates an accumulation of materials of which
many are known to be colloidal in nature, and the larger freezing-point depression suggests a higher concentration of the solution within the cells. Both conditions add to the capacity of a tissue to hold water against freezing and probably function together in this respect to a limited extent.

From the percentage decrease in water content, shown in Table I, it is not possible to arrange the varieties in order of their hardiness. Nor does the percent of increase on a green weight basis, in Table II, allow for an exact arrangement in a hardiness scale. Neither of these values indicates an exact hardiness order, but the relationship between the two may form a basis for arrangement in order of hardiness.

Some irregularities within a variety are evident in the data in Tables I and II. No suggestion is offered as to the reason for these apparent inconsistencies.

It is to be noted that data for May are not included in the comparisons because shoots were taken from last season's growth.

Unfrozen Water in Shoots Held at -20°C.

In comparing the varieties for any single determination at -10°C. it can be seen that at no time was it possible to arrange the varieties on a hardiness scale by the percentage of unfrozen water remaining in the tissue. It was thought that perhaps this temperature was not sufficiently low to actually test the ability of the shoots to withstand cold. Even the
most tender variety tested is not killed under ordinary conditions by winter temperatures as high as \(-10^\circ\text{C}\). In order to subject the tissue to a test that would more nearly approximate killing winter temperatures the shoots were frozen at \(-20^\circ\text{C}\).

In Table III comparisons of unfrozen water at \(-20^\circ\) are shown for three determinations. More determinations were to be made at this temperature, but breakdown in the freezing apparatus made it impossible to secure any data between October 29 and January 9.

**Table III**

Unfrozen Water in Shoots Held at \(-20^\circ\text{C}\). for 4 Hours

<table>
<thead>
<tr>
<th>Variety</th>
<th>October 14</th>
<th></th>
<th>October 29</th>
<th></th>
<th>January 9</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>percent</td>
<td>percent</td>
<td>E. per g.</td>
<td>percent</td>
<td>percent</td>
<td>E. per g.</td>
</tr>
<tr>
<td></td>
<td>water</td>
<td>unfrozen</td>
<td>E. unfrozen</td>
<td>water</td>
<td>unfrozen</td>
<td>E. unfrozen</td>
</tr>
<tr>
<td>Hibernal</td>
<td>48.2</td>
<td>45.4</td>
<td>.224</td>
<td>48.0</td>
<td>46.6</td>
<td>.226</td>
</tr>
<tr>
<td>Virginia</td>
<td>47.2</td>
<td>45.4</td>
<td>.215</td>
<td>47.0</td>
<td>44.6</td>
<td>.209</td>
</tr>
<tr>
<td>Wealthy</td>
<td>49.9</td>
<td>40.8</td>
<td>.195</td>
<td>49.6</td>
<td>40.1</td>
<td>.199</td>
</tr>
<tr>
<td>Jonathan</td>
<td>49.7</td>
<td>37.8</td>
<td>.181</td>
<td>49.6</td>
<td>36.3</td>
<td>.180</td>
</tr>
<tr>
<td>Delicious</td>
<td>53.1</td>
<td>35.7</td>
<td>.186</td>
<td>49.2</td>
<td>38.0</td>
<td>.187</td>
</tr>
<tr>
<td>Grimes</td>
<td>53.7</td>
<td>30.2</td>
<td>.161</td>
<td>49.7</td>
<td>35.4</td>
<td>.184</td>
</tr>
<tr>
<td>Stayman</td>
<td>51.2</td>
<td>35.9</td>
<td>.188</td>
<td>48.4</td>
<td>36.5</td>
<td>.189</td>
</tr>
</tbody>
</table>
Quantities of water unfrozen in the tissue subjected to 
-20°C. indicate somewhat more of a positive correlation than 
was found at -10°C. between percentage of unfrozen water and 
hardiness of the variety. With the exception of Stayman, which 
is considered to be very tender as compared with the other 
vvarieties, these figures might be used to separate hardy from 
tender varieties. Under such a classification Stayman would 
be among the hardy, which is contrary to practical experience 
with the variety.

Excluding Stayman, on October 14 the varieties could be 
arranged in order of their hardiness by the percent of water 
unfrozen; Hiberna1 having 15.2 percent more than Grimes on that 
date. In the other two determinations one or more of the varie-
ties are not in line with such an arrangement, but in every 
case the two more hardy varieties remain at the top of the list 
with the largest percentage of unfrozen water.

Comparisons in unfrozen water between October and January 
9 point to the fact that there is a substantial increase in 
every variety. This would suggest that reactions are taking 
place in the shoots which influence their water retaining 
capacity against freezing during the dormant period. That the 
vvarieties arrange themselves in a different order on the three 
dates, with respect to their unfrozen water, lessens the value 
of comparisons made on any single date.

It is interesting to note the exceptionally large percent-
age increase in unfrozen water of Stayman from October 14 to January 9. This emphasizes again the peculiar behavior of this variety and may indicate that maturity takes place at a later date than in the other varieties.

Unfrozen Water in Tips and Bases of Shoots Held at -20°C.

Throughout the investigation duplicate samples failed to check closely in both total and unfrozen water content. This failure was at first thought to be due to errors in weighing or temperature readings during a test. Later the idea was suggested that varying quantities of tip and base tissue in the duplicate samples might account for this difference. To find whether or not any large differences in percentage of water and percentage of water unfrozen did exist between the two portions of the same shoots they were tested separately. Table IV contains data of the results of such a test made January 9, at a freezing temperature of -20°C.
Table IV

Unfrozen Water in Tips and Bases of Shoots

Held at -20°C. for 4 Hours on Jan. 9

<table>
<thead>
<tr>
<th>Variety</th>
<th>Bases</th>
<th>Tips</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% water</td>
<td>% water unfrozen</td>
</tr>
<tr>
<td>Hibernal</td>
<td>43.1</td>
<td>64.9</td>
</tr>
<tr>
<td>Virginia</td>
<td>44.8</td>
<td>53.4</td>
</tr>
<tr>
<td>Wealthy</td>
<td>47.2</td>
<td>49.0</td>
</tr>
<tr>
<td>Jonathan</td>
<td>45.2</td>
<td>49.7</td>
</tr>
<tr>
<td>Delicious</td>
<td>45.2</td>
<td>49.3</td>
</tr>
<tr>
<td>Grimes</td>
<td>45.0</td>
<td>50.5</td>
</tr>
<tr>
<td>Stayman</td>
<td>43.0</td>
<td>56.5</td>
</tr>
</tbody>
</table>

An examination of Table IV shows that in every variety there is a higher percentage of water unfrozen in the bases than in the corresponding tips. The fact that the tips of a shoot are often killed by cold that does not seriously affect the base points again to the relationship between unfrozen water and winter hardiness. The higher percentage of total water in the tips is not alone responsible for the larger percentages of unfrozen water, because in every variety except Jonathan there is more water remaining unfrozen in the bases.
than in corresponding tips. No reason is suggested as to why
Jonathan is peculiar in this respect. It is realized that the
data are hardly sufficient to draw any well founded conclusions,
yet they do indicate the relationship that possibly exists.

Unfrozen Water in Shoots Held at -10°C and -20°C.

If winter injury is the result of desiccation of the proto-
plasm by the formation of ice within the tissue, lower temper-
atures should, under the same conditions, leave less water un-
frozen than higher temperatures. That such was actually the
case can be seen in Table V, which gives the percentage and
grams per gram green weight of water unfrozen in duplicate
samples tested January 9, at -10°C and -20°C.
### Table V

Unfrozen Water in Shoots Held at -10°C.
and -20°C. on Jan. 9

<table>
<thead>
<tr>
<th>Variety</th>
<th>Water Unfrozen</th>
<th>% per g.</th>
<th>Water Unfrozen</th>
<th>% per g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hibernal</td>
<td>56.1</td>
<td>.258</td>
<td>57.6</td>
<td>.239</td>
</tr>
<tr>
<td>Virginia</td>
<td>60.5</td>
<td>.265</td>
<td>50.8</td>
<td>.250</td>
</tr>
<tr>
<td>Wealthy</td>
<td>53.9</td>
<td>.250</td>
<td>45.5</td>
<td>.246</td>
</tr>
<tr>
<td>Jonathan</td>
<td>54.8</td>
<td>.254</td>
<td>46.5</td>
<td>.229</td>
</tr>
<tr>
<td>Delicious</td>
<td>58.3</td>
<td>.269</td>
<td>45.3</td>
<td>.215</td>
</tr>
<tr>
<td>Grimes</td>
<td>57.1</td>
<td>.260</td>
<td>45.1</td>
<td>.211</td>
</tr>
<tr>
<td>Stayman</td>
<td>57.7</td>
<td>.253</td>
<td>50.6</td>
<td>.232</td>
</tr>
</tbody>
</table>

Here is shown a rather wide range of differences in the amount of water held against freezing at the two temperatures. In every case the lower temperature froze out more water than was removed by the higher temperature. In such a test it might be assumed that the harder varieties would show a smaller difference in the quantity of water unfrozen between the two temperatures. No such relationship at this season is indicated by the figures, however.
Sap Expression Under Pressure

By subjecting tissue to physical pressure it should be possible to measure the water retention capacity of the colloids. The influence of the cell sap concentration would not be effective under this treatment. Values obtained by such a measure would not only include the water held in the living colloidal system but also that necessary for hydration of all the tissue in general. If the amount of water held by killed tissue, in which the colloidal system had been broken down by heating, were subtracted from the water held (under the same treatment) while the tissue was still alive, some indication of the importance of the water retention capacity of the colloids should be obtained. The difference in percent of water held under these two treatments is shown in Table VI.
### Table VI
**Sap Expressed Under a Pressure of 5,000 Pounds Per Square Inch**

<table>
<thead>
<tr>
<th>Variety</th>
<th>Not Frozen</th>
<th>Frozen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water held</td>
<td>Dry material in sap</td>
</tr>
<tr>
<td>Hibernal</td>
<td>46.1</td>
<td>52.9</td>
</tr>
<tr>
<td>Virginia</td>
<td>47.0</td>
<td>50.8</td>
</tr>
<tr>
<td>Wealthy</td>
<td>48.8</td>
<td>44.2</td>
</tr>
<tr>
<td>Jonathan</td>
<td>49.8</td>
<td>50.0</td>
</tr>
<tr>
<td>Delicious</td>
<td>49.5</td>
<td>46.6</td>
</tr>
<tr>
<td>Grimes</td>
<td>49.3</td>
<td>44.8</td>
</tr>
<tr>
<td>Stayman</td>
<td>48.6</td>
<td>48.3</td>
</tr>
</tbody>
</table>

**December 14, 1930**

| Hibernal | 47.6 | 48.6 | 14.8 | 23.1 | 17.5 | 5.8 | 46.9 | 52.2 | 15.0 | 24.4 | 17.8 | 6.6 |
| Virginia | 47.5 | 53.2 | 11.6 | 25.1 | 17.5 | 7.6 | 46.2 | 46.7 | 11.7 | 22.5 | 18.5 | 4.2 |
| Wealthy | 49.4 | 46.1 | 12.6 | 22.8 | 18.6 | 7.2 | 49.3 | 46.0 | 12.1 | 22.7 | 18.2 | 4.2 |
| Jonathan | 49.7 | 52.3 | 14.4 | 26.0 | 18.5 | 9.5 | 50.4 | 44.2 | 15.4 | 22.3 | 18.1 | 4.2 |
| Delicious | 50.6 | 51.3 | 10.0 | 25.9 | 16.9 | 9.0 | 50.2 | 47.2 | 10.0 | 25.7 | 17.0 | 5.8 |
| Grimes | 51.2 | 50.6 | 9.9 | 25.9 | 17.9 | 8.0 | 53.2 | 39.2 | 14.7 | 20.8 | 18.5 | 2.3 |
| Stayman | 47.7 | 55.8 | 12.3 | 25.6 | 15.5 | 7.9 | 49.5 | 49.3 | 12.9 | 21.4 | 17.3 | 4.1 |

**December 16, 1930**

| Hibernal | 45.2 | 53.0 | 18.2 | 22.4 | 19.1 | 3.3 | 44.0 | 59.2 | 15.1 | 26.5 | 21.5 | 5.0 |
| Virginia | 46.0 | 57.5 | 15.5 | 26.6 | 22.4 | 2.7 | 46.1 | 56.7 | 14.3 | 26.1 | 25.6 | 5.3 |
| Wealthy | 47.3 | 49.2 | 13.9 | 23.1 | 19.9 | 3.2 | 48.1 | 50.2 | 13.9 | 24.1 | 20.4 | 5.7 |
| Jonathan | 48.2 | 56.6 | 15.3 | 24.3 | 18.3 | 6.0 | 48.5 | 45.3 | 16.4 | 23.5 | 20.3 | 3.0 |
| Delicious | 48.1 | 52.0 | 11.9 | 25.0 | 23.1 | 1.9 | 47.9 | 61.0 | 15.0 | 24.4 | 23.4 | 1.0 |
| Grimes | 48.8 | 55.0 | 12.5 | 22.2 | 18.0 | 4.2 | 49.4 | 44.0 | 11.9 | 22.6 | 17.2 | 5.6 |
| Stayman | 47.1 | 56.2 | 14.1 | 24.0 | 16.6 | 7.4 | 49.1 | 44.1 | 15.3 | 21.8 | 19.7 | 2.1 |
Measurements shown in Table VI were all made on 30 gram samples which were subjected to a uniform treatment of 5,000 pounds per square inch for a period of one minute.

The water held against expression after readsoorption by the dead tissue is calculated as a percentage of green weight to place it on a comparable basis with water held in the live tissue. Here it will be seen that in every case the percentage held after killing the tissue by heating is less than that held by the live tissue. The difference between the two can possibly be explained by precipitation of the colloids during drying. If this difference is a measure of the influence of the colloidal system on water retention, the hardy varieties might be expected to show the largest differences in the quantity of water held. The data do not indicate any such relationship, nor do they show any correlation between water not expressed in the green tissue and hardiness.

On the basis of the theory that death from freezing is caused by precipitation of the colloids in the protoplasm, one sample was frozen at -21°C. for 20 hours previous to pressing. If freezing precipitates the colloids in the tissue, the tender varieties should show more difference in quantity of water held against pressure, between dead and live tissue, than hardy varieties.

Comparisons between samples frozen and those not frozen yield no significant differences in quantities of water held.
It is probable that the freezing temperature was not low enough to cause any large difference in the protoplasmic system of any of the varieties tested.

It is interesting to note that the percentages of water held against pressure of 5,000 pounds per square inch and that remaining unfrozen at -20°C., as shown in Table III, are much alike. This similarity suggests that approximately one-half of the water in the tissue must be held with considerable more force than the remainder. In view of the fact that shoots are rarely killed by winter temperatures of -20°C. it is probable that life can be maintained in the protoplasm with only half of its water content remaining unfrozen.

In determinations by expression duplicate samples did not check closely and any small differences that might have existed were obscured by experimental error. It is believed, however, that any large differences would have been detected by this method with the technique employed. It should be pointed out that these determinations were made during the winter months only.

Percentage of dry content of the sap expressed indicates no apparent relationship with hardiness and varies widely between duplicate samples and between varieties.
DISCUSSION

It has been suggested that an error enters into the heat-of-fusion method as employed in this investigation. In the calculation of free water, using the formula given under the heat-of-fusion method, a single freezing point is used. In Table V it is shown that more water was frozen out at -20° than at -10°. There is a possibility that the water frozen at the two temperatures had the same freezing-point and that the lower temperature only removed more water from the colloidal system in ice formation. Unless this is actually the case, it is obvious that the freezing-point cannot be correctly expressed as a single temperature.

Because of the lack of correlation in the amount of water remaining unfrozen at -10° and -20°, and the hardness of a variety, and because there is an inconsistent variation in the differences in quantity frozen out between these two temperatures, it is believed that the measurement of unfrozen water should be made at a point more near the critical temperature of the tissue. At this temperature the water would necessarily have to be held with greater force than at higher temperatures; and only at this point could the value of such a force be tested. If the protoplasm in all varieties has the same minimum water requirement for maintenance of life, then the quantity remaining unfrozen at or near the killing temperature should
indicate the relative hardiness of a variety, and only at this
critical point would the actual resistance to cold be tested.

By finding the contraction in the dry wood it is possible
that the last method of procedure described under the dilatome-
ter could be used with some degree of success. Work with the
dilatometer was not continued, however, because of other ob-
jections. There is, for example, no assurance that part of
the ice formed in the tissue is not in spaces filled with gas
which is compressed as the water changes form. If such a con-
dition existed, a measure of the external volume increase would
not be a measure of the total increase from ice formation. The
presence of a waxy appearing substance in the lignin after a
test suggests that the composition of the tissue is probably
altered by the surrounding liquid. It is difficult to predict
just what influence this change would have upon the tissue.

It should be pointed out that the rate of freezing has
been found to be of importance in the effect of temperature on
plants. In this study the samples were frozen with a more
rapid drop in temperature than is ever encountered under natural
conditions. Because of this difference in temperature drop,
comparisons with winter temperatures of the same magnitude would
hardly be justified.

Collecting samples that will be uniform is an important
item in a study of this nature. The large differences shown
in Table IV emphasize the importance of careful sampling.
though shoots of a single season's growth are used in comparisons, considerable error may be introduced by careless sampling.

At the present time it is uncertain whether or not any practical application could be made of the percentages of unfrozen water to separate hardy from tender seedlings. The most promising procedure, suggested from the data presented in this study, consists of a measure of the increase in percentage of unfrozen water in the winter wood over that of the spring wood in a single season's growth.
SUMMARY AND CONCLUSIONS

Unfrozen water in apple shoots of known hardiness was measured at -10°C and -20°C by the heat-of-fusion method. It was found that a direct correlation existed between the percentage increase in unfrozen water from June to January and the hardiness of the varieties tested. Hardy varieties showed a larger percentage increase than tender varieties.

At no determination could the varieties be arranged in order of their hardiness by the percentage of water remaining unfrozen when subjected to temperatures of -10°C and -20°C. On October 14 such an arrangement was possible, when the shoots were frozen at -20°C, except for Stayman which would fall in the hardy class on the basis of percentage unfrozen water. This variety is ordinarily quite tender and at this station is least hardy of the varieties tested.

Attempts to use the dilatometer to measure frozen water in apple shoots indicate that this method is unsatisfactory if the tissue is to be tested several degrees below its freezing-point.

When using the hydraulic press to measure bound water in apple tissue, small differences were obscured by experimental error.
SELECTED BIBLIOGRAPHY


