UNFROZEN WATER IN APPLE SHOOTS AS RELATED TO THEIR WINTER HARDINESS[1]

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(WITH NINE FIGURES)

Introduction

In breeding apples for a region where winter hardiness is an important consideration, the desirability of having some rapid and reliable means of separating the winter hardy seedlings from more tender specimens is obvious. Any elimination that can be made in the first year or two of seedling growth is economical from the standpoint of both time and cost.

A number of different methods and procedures have been employed in an attempt to separate cold resistant plants from those less resistant to freezing temperatures. A survey of the literature related to this problem suggests that the capacity of a plant to retain its moisture against the extracting forces of freezing is associated with its ability to survive cold. With this generalization in mind the present investigation was undertaken to ascertain whether or not winter hardiness in apple shoots could be related to the quantity of water remaining unfrozen at low temperatures.

The meaning of the term "bound water" used in the literature is controversial and will, therefore, be avoided in this discussion. "Water-retaining capacity" as used subsequently refers to the capacity of a tissue to retain water in the unfrozen state when subjected to certain freezing temperatures.

Literature review

Good bibliographies and reviews of the general literature on the influence of freezing temperatures on plants may be found in the publications of CHANDLER (**4**), HARVEY (**16**), MAXIMOV (**29**), NEWTON (**34**) and ROSA (**43**). An excellent discussion of the literature more closely related to the problem of winter hardiness in apple shoots is given by HILDRETH (**18**). JONES and GORTNER (**24**) included in their paper a comprehensive summary of the work published on the effect of freezing on colloidal systems. GORTNER (**12**) considered also the water relationships in colloidal and living systems. Recently he (**11**) explained the nature and the methods of estimating bound water. Critical examination of the methods of measuring and the meaning of the term "bound water" are discussed by BRIGGS (**2**), GROLLMAN (**13**), NEWTON and GORTNER (**37**), and HILL (**19, 20**).

The literature reviewed here is of more particular interest to the study presented in the following pages. ROBINSON (**42**) showed that winter hardi-

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ness of insects was related to their ability to retain water in the unfrozen state when subjected to freezing temperatures. By the heat-of-fusion method he found that tender species were unable to increase their capacity to retain water in the unfrozen state when subjected to a temperature of – 20° C. In contrast to these, hardy species exhibited considerable increase in this capacity when hardened under the same conditions as the tender species. Using the dilatometric technique, SACHAROV (**45**) obtained results in agreement with ROBINSON, although different species of insects were studied.

From his investigations on the winter hardiness of wheat plants, NEWTON (**34**) concluded that the volume of press-juice obtainable from hardened leaves was inversely proportional to the hardiness of a variety. The quantity of hydrophilic colloids contained in the press-juice, as measured by the method of NEWTON and GORTNER (**37**), was found to be directly proportional to hardiness. By means of the dilatometer, NOVIKOV (**39**) showed that the winter wheats which were more resistant to cold contained the greater quantities of unfrozen water when subjected to a temperature of – 5.75° C. The amount of unfrozen water increased with the duration of hardening in the resistant group but very little or no increase was found in the non-resistant varieties.

LOTT (**26**), working with brambles during the dormant season, observed a direct correlation between hardiness and percentage of unfrozen water as measured with the dilatometer at – 6° C. WEIMER (**59**) concluded from his studies on alfalfa roots that unfrozen water at – 5° C. was related to the degree of hardening, but it could not be used to differentiate between hardy and non-hardy varieties. An increase in the quantity of unfreezable water during the hardening process in cabbage was brought out by ROSA (**43**), who determined the frozen water at – 3°, – 4°, – 5° and – 6° C. with the dilatometer. He concluded that the rates of decrease in percentage of freezable water coincided with the observed rate of hardening.

Materials and methods

The material used throughout this investigation consisted of shoots from 15 standard horticultural varieties of apple, *Malus sylvestris*. These varieties were selected so that degrees of hardiness from very tender to extremely hardy would be included. In descending order of their hardiness, as based upon years of field observation at this station (**27**), the varieties may be arranged as follows: Hibernal, Virginia, Shield's Crab, Dudley, Okabena, Wealthy, Ioensis, Wolf River, Cortland, Baltimore, Jonathan, Delicious, Grimes, Wagner, and Stayman.

Material subsequently referred to as "nursery shoots" consisted of shoots taken from stocks planted in 1924 and cut back to the crown each winter. Shoots of all varieties from this source averaged about 3 feet in length at the end of the growing season. The term "tree shoots" applies to tip growth of a single season from trees planted in 1926. These shoots were in general about 18 inches in length at the close of the growing season. Only 10 of the 15 varieties were available in tree form. Those not available were Hibernal, Shield's Crab, Okabena, and Ioensis. The tree and nursery material was grown in adjacent plots and unformity of soil and other conditions was considered in collecting samples.

Shoots were collected before 9:00 A.M. on the day of determination and the leaves were immediately removed. After the shoots of a variety were weighed roughly to 60 gm. they were cut into three samples of approximately 20 gm. each. Each sample was then quickly weighed on a torsion balance and transferred immediately to a stoppered test tube. As soon as the three samples of one variety were weighed, two of the tubes were placed in the cooling bath at – 20° C. and the third in a refrigerator until used in a later treatment. Each sample weighed between 20 and 20.3 gm. and consisted of pieces about 1 inch in length.

The entire growth of the current year was used in sampling, except where a smaller portion was needed to bring the sample to the desired weight. In this instance a midsection of another shoot supplied the deficiency. The necessity of this procedure in sampling was indicated by a series of preliminary determinations in which it was shown that the water relationships of tip and basal portions of the same shoot were different. The mean difference in unfrozen water between all duplicates was found to be 0.80 per cent., with one-half of the observations falling between 0.4 and 1.2 per cent.

The heat-of-fusion method was used throughout this investigation to determine the values for frozen water. The first recorded attempt to apply this method to biological tissue is attributed to MÜLLER-THURGAU (**33**). Later modifications and improvements have been made by RUBNER (**44**), THOENES (**56**), and ROBINSON (**42**). HILL (**20**), ST. JOHN (**47**), and MEYER (**31**) have suggested improvements and criticisms. A detailed description of the procedure followed in this study has been presented in two previous papers (**51, 52**).

In brief, the heat-of-fusion procedure consists of measuring the change in temperature of a known quantity of water caused by the addition of the frozen material. With the above information plus the temperature, weight, and specific heat of the material it is possible to calculate the quantity of frozen water in the sample. The frozen water value is subtracted from the total water content of the sample to ascertain the value for unfrozen water.

Experimentation

EFFECT OF SLOW AND RAPID FREEZING ON UNFROZEN WATER

It has been shown (**3, 4, 18**) that the rate of freezing is a factor in determining the degree of injury in plant tissue. Non-living gel systems are also known to be affected differently by slow and fast rates of freezing (**14, 32, 54**).

A number of trials with apple shoots in the dormant condition failed to reveal any significant difference between duplicates, one set of which was cooled from 0° C. to – 20° C. in 12 hours while the other was frozen immediately at – 20° C. Apparently the rapid rates of cooling employed here had no measurable influence on the quantity of ice found in the tissue. In this connection, however, it should be pointed out that slow temperature drop is not necessarily slow freezing because of the possibility of under-cooling. JOHNSTON (**23**) observed incipient ice formation in peach buds at –5.9° to – 8.0° C. From preliminary studies with the dilatometer it is known that solidification began in these shoots between – 4° and – 6° C.

EFFECT OF PERIOD OF FREEZING ON UNFROZEN WATER

There is some evidence (**18, 36, 40**) that prolonged freezing periods bring about injury to plants. HILDRETH (**18**) observed greater injury in apple shoots when in the hardened condition after a freezing period of 12 hours at – 43° C. than after a shorter period of 3 hours. If the increased injury is caused by greater water loss from the protoplasm through ice formation, it may be expected that more ice will form with longer exposures to the freezing temperature.

During the growing season (May 23) a water-ice equilibrium was reached in the shoots by a freezing period of 4 hours at – 20° C. In the dormant condition (Jan. 18), however, a water-ice equilibrium was not attained in a 4-hour period. In the January trials 2.8 per cent. more water was removed in 24 hours of freezing than during a corresponding 4-hour period. A statistical analysis of the data obtained proved this difference to be highly significant. The value for t was 9.77 while the highly significant value for t in this instance was only 2.97 (**10**).[2] An additional 72-hour period of freezing after the first 24 hours failed to freeze more water from the tissue.

Evidently the time required for the attainment of a water-ice equilibrium at – 20° C. varied with the condition of the tissue. When the shoots were in the hardened state more time was necessary for the establishment of this equilibrium than when it was more readily killed by cold. Perhaps the longer freezing time required to reach equilibrium in the shoots while in the hardened condition is a factor in their surviving cold. The influence of time is important in a study of heat treatments on colloidal and living systems and it is possible that death from cold is a time-temperature relationship as is death from heat.

[2] t is a value used in the test for significance of differences between means. See FISHER (**10**).

The percentage of moisture in the tissue is also probably a factor in determining the time for attainment of equilibrium. JONES and GORTNER (**24**) found that the time to reach a water-ice equilibrium in gels varied with the concentration and the temperature of exposure. At temperatures near the freezing point the rate of reaching equilibrium was slower than at colder temperatures. Gels of higher concentration froze more slowly than those with less dispersed material.

In this respect the behavior of apple shoots resembles that of gels in that it requires more time to reach a water-ice equilibrium when the moisture content is lower. On the other hand, observations in apple shoots frozen at – 5° C. proved that equilibrium at this temperature was more rapid than at the colder temperature of – 20° C. This is just the reverse of the behavior with gels as observed by JONES and GORTNER (**24**).

INFLUENCE OF COLD TEMPERATURES ON WATER-RETAINING SUBSTANCES

To measure the behavior of apple shoots when subjected to a previous cold temperature and then frozen at a milder temperature, a sample was cut in triplicate. Two of the three were frozen at – 20° C. for 4 hours and then the freezing bath was allowed to warm to – 5° C. When the latter temperature was reached one set of samples was taken from the bath and placed in a water bath at room temperature for one hour. At the end of the hour this set of tubes was returned to the – 5° C. bath along with a triplicate not yet frozen. All three sets were then frozen 14 hours at – 5° C.

The close similarity of the triplicate samples in table I is evidenced by a comparison of the percentages of water within a variety.

TABLE I

INFLUENCE OF PRECEDING LOW TEMPERATURES ON UNFROZEN WATER. MAY 3, 1933

VARIETY	TREATMENT					
	I		II		III	
	– 5° C. ONLY		– 20° C. THAWED		– 20° C. NOT THAWED	
	WATER	UNFR. WATER	WATER	UNFR. WATER	WATER	UNFR. WATER
	%	%	%	%	%	%
Hibernal	53.4	47.6	53.1	43.1	53.0	37.7
Virginia	55.0	44.0	55.3	39.6	55.3	30.8
Dudley	53.0	46.5	52.6	42.3	52.4	35.2
Wealthy	52.0	49.0	51.5	43.6	51.2	38.2
Ioensis	51.9	49.2	51.6	41.9	52.2	36.2
Delicious	53.3	46.5	52.7	43.0	53.2	33.5
Stayman	51.3	45.3	51.0	38.6	51.4	38.9
Mean	52.8	46.9	52.6	41.7	52.7	35.8

A comparison of the values for unfrozen water in treatments I and II indicates that the prefreezing at – 20° C. caused some alteration in the tissue that impaired its water-retaining capacity against subsequent freezing at – 5° C. This alteration is shown by the fact that the set frozen at – 5° C. only was able to hold 46.9 per cent. of the water unfrozen, while the set previously cooled at – 20° C. could hold unfrozen only 41.7 per cent. of the water. Thus it is seen that the process of freezing out and reabsorption of water by the tissue is not reversible.

Evidence of a partial reversibility, however, is obtained from the fact that part of the water originally frozen at – 20° C. was reabsorbed and not frozen out again by the – 5° C. temperature. This is clear from a comparison of the unfrozen water values in treatments I, II, and III. Here it is seen that the mean value for unfrozen water in the – 20° C. thawed set (41.7) is approximately midway between the means of the – 20° C. not thawed set (35.8) and the – 5° C. only set (46.9). If all of the water originally frozen at – 20° C. in the thawed samples had been formed as ice again in the refreezing at – 5° C. it might be expected that the two means for unfrozen water in treatments II and III would be the same. Since approximately one-half of it remained unfrozen at – 5° C., a partial reversibility is evident.

JONES and GORTNER (**24**) were unable to demonstrate complete reversibility in the gelatin systems they studied. In systems of inorganic hydrogels they found the quantity of water frozen to be increased by a previous lower temperature. The behavior of apple shoots is similar to that found in inorganic hydrogels as reported by JONES and GORTNER (**24**).

The data in table I are also of interest from the view-point of winter injury. If cold injury results from a dehydration of the protoplasm by the removal of water in the formation of ice, a period of severe cold followed by warmer weather that remained below freezing would probably be as injurious as if the lower temperatures had continued. The basis for this conclusion is apparent from treatments I and III, where it is shown 11.1 per cent. less water remained unfrozen in the set previously frozen at – 20° C. than in the – 5° C. only set. The value for unfrozen water when measured at –20° C. on this date was approximately the same as the unthawed treatment at – 5° C. in table I.

Thus it would seem that once the water has been frozen it remains in the solid state at a warmer temperature than could have frozen it originally. This failure of the ice to melt indicates that colligative properties are of minor importance in retaining the water in the liquid state. If they were effective, melting of the ice could be expected at the warmer temperature. Results similar to those contained in table I were obtained with a temperature interval of – 20° and – 43° C.

UNFROZEN WATER AT – 43° C.

A freezing temperature of –20° C. was used for the most of the determinations in this study, principally because RUBNER (**44**), THOENES (**56**), and ROBINSON (**42**) have assumed that all of the free water and none of the bound water is frozen at this point. GORTNER (**12**) has stated, ". . . when a part of the bound water is removed from the hydrophilic colloid, the colloidal structure is altered and vital function interfered with."

A number of observations were made during January, 1933, at – 43° C. to ascertain whether or not additional water could be frozen out of the tissue over that removed by – 20° C. The temperature lowered from – 20° to – 43° C. brought about an average decrease of 12.5 per cent. in unfrozen water. If this additional quantity frozen out by the 23° C. drop in temperature is added to 22.5 per cent., the mean difference between – 5° and – 20° C. at this time, the total decrease in unfrozen water from – 5° to – 43° C. is 35 per cent. In other words the unfrozen or bound water decreased very decidedly with temperature lowering.

JONES and GORTNER (**24**) found the quantity of unfrozen water in gelatin gel to change very little, if at all, between – 6° and – 50° C., while in silica gel and other gels of the inelastic type unfrozen water decreased with temperature lowering. Again there is evidence that the behavior of the substances responsible for retaining water unfrozen in apple shoots is similar to the inelastic type of gel rather than to the elastic type.

From the view-point of winter hardiness – 43° C. may be a more satisfactory temperature than – 20° C. for comparison of varieties during January. HILDRETH (**18**) showed that shoots from a tender apple variety were killed by a temperature of – 41° C. in January, while a hardy variety survived this temperature. The data collected at – 43° C., however, did not allow the varieties used in this study to be properly arranged in order of their hardiness.

INFLUENCE OF A PRECEDING WARM TEMPERATURE ON UNFROZEN WATER

It is commonly observed that injury to plants from cold will result if a warm spell during winter is followed by low temperatures which would not have been injurious in the absence of the mild period. Injury of this nature is especially prevalent on peaches and plums in a region where the temperature fluctuates over a wide range during the winter. Under such conditions some alteration in the water relationships should occur if the injury is the result of water loss in the formation of ice.

To measure the influence of a warm period, as it affects the water-retaining capacity of the shoots when frozen, four shoots of each of seven varieties were collected on January 13, 1933. In two of these sets unfrozen water was measured immediately. One set was placed with the cut ends in water in cold storage at 0° C., the other set remained in the laboratory at room temperature. After a storage period of 11 days unfrozen water in these last two sets was measured at – 20° C., as in the first two.

TABLE II

EFFECT OF A PREVIOUS WARM SPELL ON WATER-RETAINING CAPACITY AGAINST FREEZING AT —20° C.

VARIETY	JAN. 13, 1933				JAN. 24, 1933			
	SAMPLE I		SAMPLE II		ROOM TEMPERATURE		0° C.	
	WATER	UNFR. WATER	WATER	UNFR. WATER	WATER	UNFR. WATER	WATER	UNFR. WATER
	%	%	%	%	%	%	%	%
Hibernal	47.5	43.6	46.3	41.5	53.3	37.9	51.4	37.7
Virginia	51.6	36.0	51.0	37.5	57.0	27.1	53.9	34.8
Dudley ...	50.2	39.4	45.8	45.5	56.1	29.7	51.5	38.9
Wealthy	47.4	41.7	47.4	45.2	54.8	29.3	50.8	40.2
Ioensis ...	48.8	41.2	47.4	41.9	53.6	32.1	52.5	38.7
Delicious	45.7	42.6	50.2	35.5	56.4	30.5	52.6	36.3
Stayman	46.7	39.8	47.1	42.0	54.0	30.4	52.2	36.4
Mean...	48.3	40.6	47.9	41.3	55.0	31.0	52.1	37.6

Comparison of the data given in table II in terms of t is as follows:

COMPARISONS	VALUES OF t
Sample I and sample II	0.50
Room and sample II	4.75
0° and sample II	0.53
Significant	2.14
Highly significant	2.98

In the data presented in table II the accuracy of replication in sampling is observable from a comparison of samples I and II. No difference of statistical significance was found between these two sets.[3] Similarly the samples stored at 0° C. were not significantly different from set II in grams of water held unfrozen. The samples held at room temperature, however, showed a decided decrease in water-retaining capacity when frozen at – 20° C. The highly significant value for t of 4.75 is evidence of this fact. It is probable that a warm spell in winter brings about a similar change in

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[3] Tests of significance of differences in these comparisons were based on grams of unfrozen water rather than percentages because of the increase in total water in the stored samples.

the water relations of shoots, and, if true, the association of water-retaining capacity with ability to survive cold becomes apparent.

UNFROZEN WATER AS A MEANS OF SEPARATING HARDY AND TENDER VARIETIES

To obtain a knowledge of the changing water relations throughout the year, determinations were made on all varieties at monthly intervals. By this procedure the rate and time of maturity, as indicated by water relationships, would be disclosed together with the relative water-retaining capacities of the different varieties.

In figures 1, 2, and 3 may be seen the course of the total water and the unfrozen water held at – 20° C. throughout the year. The three varieties selected for figures 1, 2, and 3 represent a very hardy variety (Virginia), a very tender variety (Stayman), and one intermediate in its ability to survive cold (Wolf River), as observed from years of horticultural field experiences at the Iowa Agricultural Experiment station (**27**).

Although there is a difference in the course of the curves of the three varieties, no single variety maintains its relative position consistently throughout the year. The interlacing of these curves is characteristic of all the fifteen varieties tested. The relative position of the varieties changed from month to month and at no time could a hardy variety be distinguished from a tender one by the percentage of unfrozen water in each. Not only was this true when unfrozen water was expressed as a percentage of the total water, but also when expressed as a percentage of the dry weight, as a percentage of fresh weight, or in grams of water.

STATISTICAL ANALYSIS OF VARIETAL DATA

Even though it is impossible to separate varieties into an order of hardiness, a statistical analysis of variance indicates that varieties differ significantly in their capacities to retain water against freezing. Results of the analysis are shown for the three shoots as well as for the nursery shoots for both years (table III).

In the test for significance of varietal differences it is necessary to compare the value (1429) listed under "mean square" with that of "interaction" (203) in the same column. In like manner the significance of the difference between months may be tested. By these tests there was found in all three series a highly significant difference between varieties. Tests for significance were made in accordance with FISHER's method, but using the tables for "F" given by SNEDECOR (**50**). This is statistical evidence that varieties differ significantly with respect to their water-retaining capacity against freezing, although it is not possible to arrange them in an order of hardiness on this basis. In spite of the fact that this analysis does not explain at what time or where the differences occur, it does prevent

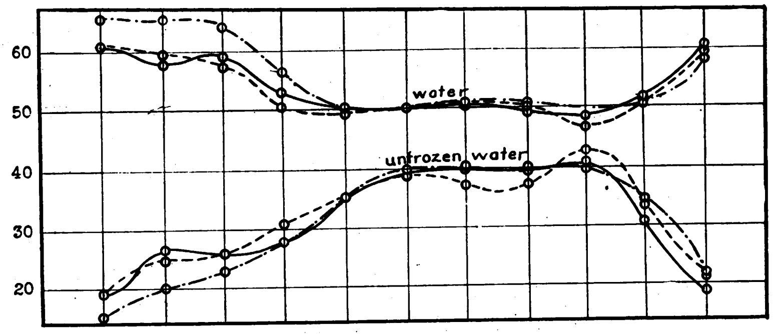

FIG. 1. Percentage of total and unfrozen water, 1931–32.

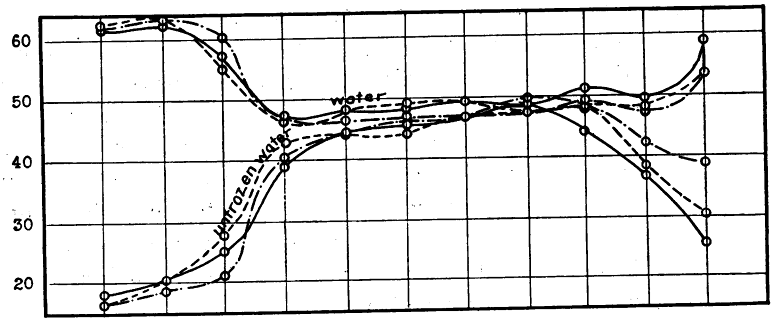

FIG. 2. Percentage of total and unfrozen water, 1932–33.

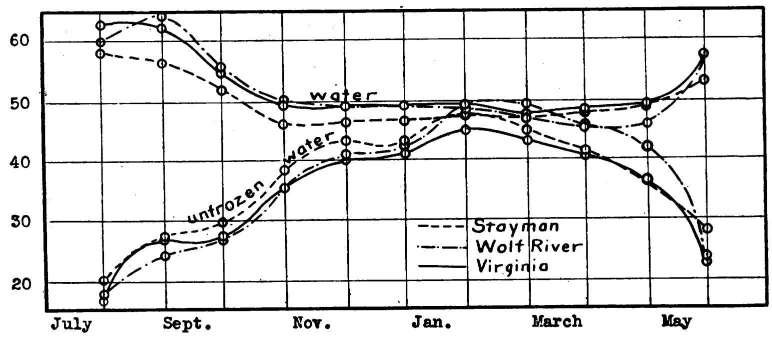

FIG. 3. Percentage of total and unfrozen water in tree shoots, 1932–33.

TABLE III

ANALYSIS FOR VARIANCE IN VARIETIES TESTED

VARIANCE	NURSERY SHOOTS				TREE SHOOTS	
	1931–32		1932–33		1932–33	
	D.F.	MEAN SQUARE	D.F.	MEAN SQUARE	D.F.	MEAN SQUARE
Total	329	1412	359	2612	219	2375
Within pairs	165	70	180	107	110	139
Between means of varieties	14	1429	14	920	9	1811
Between means, months	10	40463	11	76663	10	44977
Interaction or experimental error	140	203	154	404	90	431

the mistake of concluding that varieties are all alike in this respect. The difference in unfrozen water between months is more obvious than that between varieties and is also proven highly significant by the same test. This latter significance would be expected from an inspection of the curves.

Errors of technique and sampling are shown in table III. Values listed after "within pairs" are indicative of the error resulting from laboratory technique alone, while those under "interaction" represent the biological error plus the error of laboratory technique. This latter combination is the best estimate of the "experimental error." SNEDECOR (**50**) has discussed in some detail the calculation and the significance of these two errors.

Perhaps these results of varietal comparisons should not be surprising, in view of the fact that DUNN (**8**) and HILDRETH (**18**) found the survival order among varieties to change from time to time when apple shoots were frozen and allowed to recover. Even so, NICHOLS and LANTZ (**38**) and DORSEY (**6**) have observed in apple trees that the degree of browning after winter injury is quite consistent for a variety. It should be pointed out, however, that the degree of browning is not necessarily a measure of survival since many shoots may be severely browned by the cold and yet recover apparently uninjured.

A difference will be noted between water relationships in the same three varieties when taken from the trees and from the nursery row (figs. 2, 3). This difference is probably brought about by environment and dissimilar cultural practices, and suggests that environmental as well as inherent characteristics are important in determining water relationships in the shoots.

Another basis for comparison of varieties was suggested by the work of ROBINSON (**42**) who found that it was the increase in capacity to retain water against freezing, after a hardening treatment, that distinguished a hardy from a tender species of insect. A comparison of the increase in unfrozen water values from July to January failed to reveal any differences that could be used to separate resistant from non-resistant apple varieties.

COMPARISON OF VARIETIES AT – 5 TO – 20° C.

It has already been shown that the formation of ice in apple shoots increased with temperature lowering. Such a relationship indicates that part of the water freezes readily, while other portions form as ice only at lower temperatures. Further cooling, after the first part is frozen, would freeze that water which is retained more tenaciously and is removed only by the colder temperature. A test of the capacity of a shoot to retain water between a high and a low freezing temperature should give some measure of its ability to survive cold, since it is this last portion, held more firmly, that decides survival or death through freezing.

In determining the quantity of water removed between a mild and a more severe temperature, unfrozen water measurements were made in duplicate samples held at – 5° and – 20° C.

TABLE IV

MEAN DIFFERENCES IN UNFROZEN WATER BETWEEN – 5 AND – 20° C.

VARIETY	ORDER OF HARDINESS	MEAN DIFFERENCE IN PERCENTAGE OF WATER UNFROZEN	ORDER OF DIFFERENCES
Hibernal	1	12.0	8
Virginia	2	10.7	3
Shield's	3	9.9	1
Dudley	4	11.1	5
Okabena	5	10.9	4
Wealthy	6	11.6	6
Ioensis	7	9.9	2
Wolf River	8	11.8	7
Cortland	9	13.4	11
Baltimore	10	13.8	12
Jonathan	11	14.9	14
Delicious	12	13.0	10
Grimes	13	13.9	13
Wagner	14	12.1	9
Stayman	15	14.9	15

The mean difference column in table IV was derived from an average of four monthly determinations in 1932–33. Data of December, January, February, and March were used in this comparison. These months were selected because they cover the period of greatest resistance to cold.

It is obvious from the data that the varieties cannot be arranged in the proper order of hardiness. If a line is drawn under the value for Wolf River, however, it may be seen that varieties falling below the line have larger mean differences than those above. With the exception of Hibernal and Wagner the varieties fall definitely into two distinct groups. Comparing the two groups statistically gave a value for t of 5.41 with 3.01 as the highly significant value. This is statistical evidence that during the dormant season a tender variety can be distinguished from a more resistant one by the larger quantity of ice formed in its shoots in the 15° interval between $-5°$ and $-20°$ C.

The arbitrary position of the line of separation should not be overlooked in drawing conclusions from these data.

COURSE OF WATER RELATIONSHIPS THROUGHOUT THE YEAR

For a general discussion of the course of the water relationships throughout the year the means of the 15 varieties are plotted in figures 4 and 7. Charts for the temperature and rainfall over the same period accompany these curves. Rainfall is illustrated as total precipitation per week, while the points on the temperature curves are determined as means of 10-day intervals for maximum and minimum temperatures as recorded by the United States Weather Bureau at Ames, Iowa.

The position of each point in figures 4 and 7 is determined by the mean of 15 varieties. With this number of observations a small difference in location of the point is likely to be of more significance than if the position were established by a single pair of observations of one variety.

These curves represent data from shoots of a single growing season. All new growth present in the spring was removed before the samples were cut. In this way the changes observed were not attributable to the presence of new succulent tissue but rather to alteration of the material of the same source as that studied through the preceding summer and winter. The unfrozen water was measured at $-20°$ C.

On most varieties buds were just beginning to enlarge on April 21 in 1933, and leaves were only partly developed on April 21, 1932. As observed from material in the field there is apparently no relationship between the hardiness of a variety and the time its buds open in the spring. Nevertheless the stage of development in the buds is reflected by the water relations in the shoots. The data obtained on April 21, 1933, showed that varieties having buds ready to open retained a smaller percentage of water unfrozen than those varieties exhibiting a less advanced stage of bud development.

This alteration in water-retaining capacity with spring growth is probably best shown by the abrupt drop in unfrozen water from March to April (fig. 7). This sudden change in water-retaining capacity against freezing

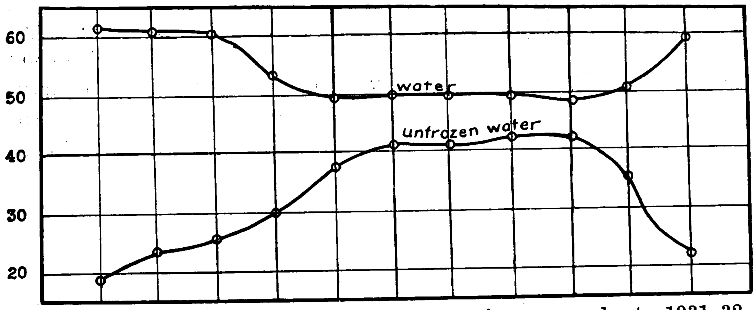

FIG. 4. Percentage of total and unfrozen water in nursery shoots, 1931–32.

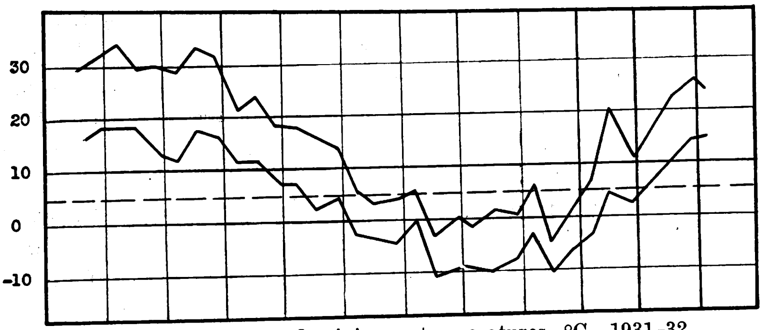

FIG. 5. Maximum and minimum temperatures, °C., 1931–32.

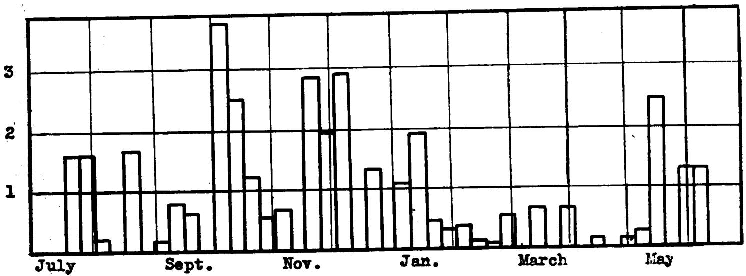

FIG. 6. Precipitation in inches, 1931–32.

takes place in spite of a slight decrease in the total quantity of water present. A similar decrease in percentage of water unfrozen is also noticeable in the spring of the previous year (fig. 4).

Another change in the water-retaining capacity of the tissue is noticeable in the autumn and early winter of each year. In early winter this change also takes place quite independently of the total water content of the shoots. Undoubtedly the cessation of food synthesis took place long before November, when many of the leaves had fallen and the remainder were dead from frost. Still, in both years there was a gradual increase in capacity to retain moisture unfrozen until March. This increase proceeded even after the water content in the tissue had reached the constant level maintained throughout the winter.

With the exception of the two examples just discussed the quantity of water remaining unfrozen appears to be rather closely related to the total water content of the tissue. This is shown by the fact that a low percentage of unfrozen water usually accompanies a high moisture content, while a high water-retaining capacity characterizes shoots having a low percentage of water. If the unfrozen water were merely a function of the total quantity present, it would remain unchanged after the water content of the shoots reached a constant level. In general this is the case, but the two exceptions noted in the spring and autumn furnish evidence that there must be some other influence on water-retaining capacity besides the percentage hydration of the tissue. In the autumn the exception is an increase while in the spring it is a decrease in capacity. These exceptions indicate that at least part of the substances retaining water unfrozen are associated with the vital or chemical activities of the tissue. If such were not the case it would be difficult to account for these alterations in capacity without an accompanying change in quantity of matter present. It is concluded, therefore, that living processes have an influence upon the substance responsible for retaining water unfrozen. This conclusion is contrary to observations of MEYER (**31**) on the leaf tissue of pine.

Of course it is impossible from the data on hand to account for the reactions responsible for this alteration in water-retaining capacity. It is suggested, however, that the protective action of sugar may be of some importance in this respect. Especially, in the light of a recent publication by NEWTON and BROWN (**36**), does the influence of sugar seem possible. They found in freezing expressed plant juices that less precipitation of proteins occurred after sugar had been added than when it was omitted. If such protective action existed in apple shoots, one might expect a maximum in water-retaining capacity during the time when sugar is known to be present in largest quantities. In an analysis of apple shoots HILDRETH (**18**) discovered that the highest sugar content occurred during the winter months. At this time the water-retaining capacity against freezing is at a maximum, as seen in figures 4 and 7. The occurrence of the two maxima at the same time may be a mere coincidence, but there is a possibility of an interrelationship.

Along with the protective action of sugar it is possible that alterations in the materials present may cause some change in water-retaining capacity. In autumn, synthesis of substances into more complex structures could alter the physical and chemical state of the matter present without appreciably changing the dry weight. In the spring these same substances could be changed in the reverse direction by an analysis or breaking down process. This change in the state of the matter on hand could account for the alteration in water-retaining capacity without a measurable increase or decrease in percentage of dry matter.

Some unpublished observations of MARTIN (**28**) are of interest here. In a study of hardiness of sweet clover over a number of years he has found repeatedly, through cytological technique, that the protoplasm in the cells of the roots appeared very dense at the approach of winter. With the inception of growth in the spring he observed an abrupt liquefaction of the protoplasm. The exact time at which these changes took place varied with the weather conditions in late autumn and early spring. LOOMIS (**25**) has suggested that hardiness is the result of a structural differentiation of the protoplast which makes it more resistant to precipitation, such differentiation being dependent upon, and in part initiated by, a high sugar concentration in the tissue. These observations appear to be in agreement with the results of this study, but it should be mentioned, in opposition to this view, that HARVEY (**16**) believes the process of hardening is accompanied by an analysis rather than a synthesis of substances. He found more decomposition products of proteins in cabbage after hardening than in the unhardened leaves. NEWTON and BROWN (**36**), however, were inclined to attribute the presence of such intermediate products to the action of freezing and not to the hardening treatment.

No matter what the explanation underlying the changes in water-retaining capacity may be, it seems clear that it is associated with, or at least parallels, the hardiness of the shoots. HILDRETH (**18**) has plotted killing temperatures for apple shoots over the course of a year. His data show a killing temperature of – 3° C. in July, with a gradual lowering to about – 40° C. on the first of January. Little change is then noted until an abrupt upward trend begins the first part of April, reaching – 6° C. in May. The striking relation between HILDRETH's killing temperature curve and the curves for unfrozen water in this study suggests that the ability to retain water against freezing may be of importance in cold-survival of apple shoots.

The greater resistance during the winter months is associated with a larger water-retaining capacity at this time. The same freezing temperature is able to remove as ice a much smaller percentage of water during the period of greatest resistance than when the tissue is more readily killed by cold. This smaller portion removed as ice would mean less departure from the normal water relationships in the tissue. It may be that it is necessary to remove as ice a rather definite percentage of the total water present before serious injury or death of the protoplasm results. Under such a condition the shoots in the winter could withstand a much lower temperature before this minimum percentage would be reached.

Too much emphasis should not be placed on the percentage of water unfrozen without a discussion of the percentage of total water. Numerous investigators (**1, 7, 23, 35, 55**) have pointed out that hardiness is associated with a low moisture content. Some authors (**22, 48**) have contended that hardy varieties can be separated from tender ones by their lower moisture content. Such is not the case with the apple shoots used here, although there is evidence that the degree of resistance to cold is related to the water content of the tissue. This relationship is readily seen from an inspection of the percentage water curves in figures 4 and 7. From summer to winter there is a marked decrease in the moisture content of the shoots. During the winter the percentage of water reaches a low level that remains quite constant until spring, when it rises very abruptly. It is obvious that when the shoots were least resistant the moisture content was high, while during the period of great resistance to cold the percentage was low.

A comparison of the two years shows that the percentage of water in the shoots was higher during the winter of 1931–32 than in the following year. A lower percentage of unfrozen water is also observable in 1931–32. The fact that the two years differ with respect to the position of their curves is probably correctly explained by the comparatively warm, moist autumn of 1931, in which some woody plants blossomed in later fall and many exhibited a renewal of growth at this time. A glance at the temperature and rainfall charts (figs. 5, 6, 8, 9) shows the difference between the two autumn seasons.

From past experience with winter injury to fruit trees it is known that the conditions of 1931 were not conducive to a thorough hardening as contrasted to the weather of 1932. DEXTER (**5**) and TUMANOV (**57**) have found recently that conditions favoring food accumulation are generally favorable for the hardening of plants. The season of 1931 was not especially favorable for the accumulation of foods as compared with the following autumn. It might be assumed, then, that the shoots were less hardy during this year than during the following year; an assumption that is supported by the smaller percentage of unfrozen water in the shoots.

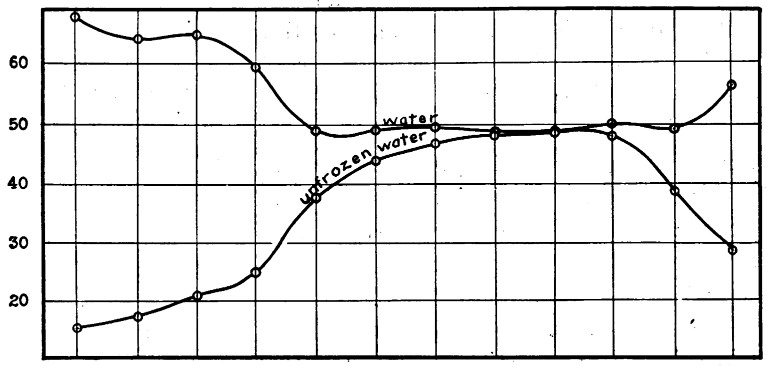

FIG. 7. Percentage of total and unfrozen water in nursery shoots, 1932–33.

FIG. 8. Maximum and minimum temperatures, °C., 1932–33.

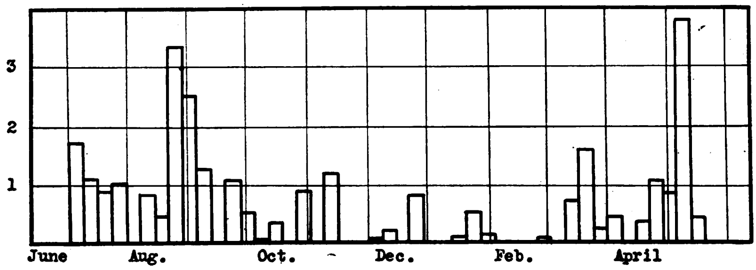

FIG. 9. Precipitation in inches, 1932–33.

Another relationship between the course of the curves for unfrozen water and climatic conditions is suggested by an investigation by HARVEY (**17**). In a study of elm seedlings he determined the threshold value for hardening to be about 5° C., when the exposure was continuous. Little or no hardening was observable at 10° C. In figures 5 and 8 it may be noted that the 5° line intersects the maximum temperature curves in the spring of both years at about the same time the drop in unfrozen water occurs. The influence of a warm temperature on the ability to retain water unfrozen was pointed out in a previous section and those results are apparently substantiated by a similar decrease observable here.

Discussion

In attempting to separate apple varieties into an order of hardiness many factors must be considered. First, the order of hardiness probably varies from time to time, with no variety maintaining the same relative position throughout the year. Not only is there a variation between varieties but the shoots of the same variety also respond differently to the same freezing temperature. Different degrees of injury through cold may occur without death resulting to the entire shoot. It is only when a sufficiently large portion of the living tissue is killed that recovery becomes impossible. The magnitude of this portion may vary among varieties and from shoot to shoot within a variety.

Again there is a difference in the same variety brought about by the external environment. The hardiness of a tree will vary with climatic and other conditions quite independently of its inherent resistance to cold.

In spite of these possible sources of variations, hardy varieties survive winters that kill the more tender sorts. It is this constancy of behavior that suggests some inherent difference in the hardiness of the varieties.

To test this inherent difference, by measuring the capacity to retain water unfrozen, is complicated by the factors mentioned above, plus the lack of information as to the necessity of this capacity in survival. The rate of freezing and the rate of thawing have both been shown to have an influence on the injury resulting from low temperatures. Neither of these measurably affect the quantity of water frozen. There are probably other factors influencing the amount of injury without appreciably altering the quantity of water frozen in the tissue. It is these additional influences that demand caution against placing too much emphasis upon capacity to retain water against freezing as the major factor in surviving cold. Nevertheless the importance of this capacity is evidenced again and again in the study presented here. This is especially noticeable in the effect of period of exposure to freezing temperatures, in the effect of a preceding warm period, in the comparisons at $-5°$ and $-20°$ C., and in the general course of unfrozen water throughout the year.

The attempts to separate the hardy from the tender varieties using unfrozen water as a basis were futile, with perhaps the one exception of the comparison made at – 5° and – 20° C. In this case the tender varieties, taken as a group, exhibited a greater loss of water in formation of ice between the two temperatures than did hardy varieties. Although the data indicate that such a procedure might serve for placing a variety into a hardy or tender class, more evidence is necessary to substantiate this point before a positive assertion can be made.

Summary

1. Unfrozen water measurements were made on apple shoots by the heat-of-fusion method at monthly intervals throughout the year.
2. Some evidence was obtained to show that the freezing process in apple shoots was partially reversible, resembling the behavior of an inelastic gel in this respect.
3. In general, the data supported the hypothesis that the capacity to retain water against freezing is associated with winter hardiness of apple shoots.
4. A statistical analysis of the data indicated that varieties differ in their capacity to retain water against a freezing temperature of – 20° C. Nevertheless it was impossible on this basis to separate the varieties into a hardy and a tender class.

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