PRELIMINARY RESULTS IN MEASURING THE HARDINESS OF PLANTS[1]

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(WITH TWO FIGURES)

The injury of plants occasioned by winter conditions has been a subject of great interest and importance to plant breeders and agriculturists. The development of hardy strains has been, and is, one of the principal means of combating losses caused by injury from cold, although cultural methods, as well, have been adapted to help the plant survive the rigors of its climatic environment.

Recognition of the degree of hardiness has necessitated field trials commonly requiring extended periods of time, during which weather conditions may be too mild to test the relative hardiness in one season, while so severe in the next that hardy as well as tender varieties and strains are severely injured. It is quite evident that a rapid means of measuring relative degrees of resistance to cold of strains and varieties of plants, grown with various cultural procedures, would hasten progress in the work of plant breeders, as well as facilitate investigations of the physical and chemical nature of the hardening process, and its relation to the resistance of plants to low temperatures and other climatic conditions.

No attempt will be made to review the literature on hardiness or reactions of plants to freezing in reference to changes in permeability, colloidal structure or compositional variations. It seems to be generally accepted that the injury, or killing of tissue, by cold, or by any other means, involves the disorganization of the substances essential for carrying on the processes of life. With such disorganization, it is well recognized that the cell loses its capacity to regulate the diffusion of its soluble contents. Upon this basis, it was assumed that the degree of injury from low temperature, to overwintering and other plant structures might be correlated with the exosmosis of electrolytes and other materials following exposure to cold. Such outward diffusion of electrolytes can readily be estimated by conductivity measurements. Other investigators have applied conductivity methods to problems involving the viability of seeds, and the injury of plant and animal tissues.

To test this hypothesis, alfalfa roots of three varieties of known hardiness (Grimm, Utah Common, and Hairy Peruvian) were frozen under con-

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trolled conditions, for various lengths of time, and at various stages of growth throughout the autumnal season. The plants used were grown under fertile soil conditions from seed sown on July 13, 1928, in plats on the University Farm at Madison, Wisconsin. They were not cut that season, and the stands in all plats were excellent. They survived the very favorable winter season of 1928–29 without apparent injury.

During the growing season of 1929, a half of each plat was cut twice, on July 9 and September 12, when the plants were in full bloom, while the remaining half of each plat was cut four times, on June 22, July 9, August 13, and September 12. This latter treatment was one which would greatly inhibit the storage of organic foods in the roots, while the two-cutting treatment would provide for an abundance of such reserves.

Preliminary work during the early autumn showed that much more extensive outward diffusion into distilled water occurred when the roots had been frozen than when they were uninjured, but no varietal differences were observed. On September 21, 1929, a standardized procedure was first applied to this study. Roots were dug from the plats of alfalfa cut only twice and such alfalfa was therefore "high" in reserve food content. They were trimmed at the crown, to leave buds for further development, cut to a uniform length of seven inches, then washed hurriedly but thoroughly in running water, by taking a small bunch of roots and rubbing them between the hands, thus giving little opportunity for exosmosis in the washing process. The roots in bunches of several hundred were then covered with a damp cheese cloth, whereupon gradual evaporation brought the specimens to a uniform condition of surface moisture.

Samples consisting of 8 roots, trimmed at the base when necessary, to weigh 20 grams, were selected and placed in Pyrex test tubes (1 x 8 in.), closed with rubber stoppers. Five such tubes of each variety were placed in a circular rack, for each freezing treatment. The freezing was carried on in a cylindrical ice cream can of five gallon capacity, in a large thermostatically regulated electrical refrigerator, where temperatures ranged between $-8°$ C. and $-9°$ C. The roots were left at this temperature for 1.5 hours, 4 hours, and 15 hours. In each case, five tubes of roots of each variety were similarly prepared, but not frozen. Thus, 15 tubes were not frozen but were used as checks; 15 were frozen for 1.5 hours; 15 for four hours; and 15 for 15 hours. Each tube contained 8 roots, weighing 20 grams. After freezing, the roots remained in the tubes at room temperature for several hours. Three tubes of each variety from each freezing treatment were selected for transplanting in the greenhouse, where their recovery and subsequent growth might be observed. The two remaining tubes of each variety, from each freezing treatment, were placed in a rack.

This rack was suspended in a thermostatic water-bath, at 25° C., and to each tube, in succession at 2.5 minute intervals, 50 cc. of distilled water was added from a pipette. This water completely covered the roots. The tubes of roots remained in the bath for a ten-hour period.

At the expiration of this time, the liquid about the roots was withdrawn from each tube in succession, with a pipette, redischarged into the tube for mixing, and then again withdrawn and transferred to a conductivity cell, where the reading of resistance was taken to the nearest ohm. This precaution of thorough mixing appeared, upon investigation, to be unnecessary, but was nevertheless adhered to. The roots were removed from the tube and the solution was preserved for further chemical study. In this way, each tube of roots had an equal time for exosmosis into water. In several preliminary experiments it had been found that there was little change in conductivity after ten hours, and this interval was, therefore, chosen for all later experiments.

The freezing periods of 1.5 hours, 4 hours and 15 hours were chosen after some study, in the hope that the brief freezing would show slight injury, while the fifteen-hour freeze would completely kill all varieties. The one and one-half hour freeze was discontinued after four trials because the injury to the roots, as shown by the greenhouse checks, was slight in the later part of autumn. The fifteen-hour freeze was sometimes omitted to permit other experiments to be run. The four-hour freeze was run regularly at weekly intervals.

The average values for the specific conductivity, determined in duplicate in each case, and expressed in reciprocal ohms, on each date, for each variety are given in table I, and are represented graphically in figure 1. The figures presented are for the four-hour freeze.

TABLE I

SPECIFIC CONDUCTIVITIES ($\times 10^6$) IN RECIPROCAL OHMS, OF WATER EXTRACTS FROM ROOTS OF ALFALFA, AFTER FREEZING FOR FOUR HOURS. AN INTERVAL OF TEN HOURS WAS ALLOWED FOR EXOSMOSIS

VARIETY	SEPT. 21	OCT. 11	OCT. 18	OCT. 25	NOV. 1	NOV. 8	NOV. 15	NOV. 27
Grimm	1632	1250	1015	921	549	781	629	484
Utah Common	1657	1255	1235	1200	1177	1215	1270	879
Hairy Peruvian	1624	1459	1375	1535	1365	1447	1257	1475

No varietal difference was found in hardiness as measured by exosmosis from samples dug and frozen September 21, but during the latter part of October and the month of November prominent varietal differences are evident. This indicates that a very definite hardening process occurs in Grimm alfalfa during the fall period, while Peruvian has about the same resistance to cold on November 27 as on September 21. The Utah Common is intermediate throughout this period, but the hardening process seems to be delayed until the advent of freezing weather on or about November 19.

Apparently, the reaction of the plant to the climatic environment of the autumn period has much to do with its resistance to cold. The external appearance of these varieties growing under field conditions was markedly different during October and November. The last cutting was made on September 12, but owing to dry weather, there was very little growth recovery in any variety until September 28, when regeneration was stimulated by abundant rainfall. The character of this recovery varied widely in the different varieties. The growth of the Peruvian was rapid and up-

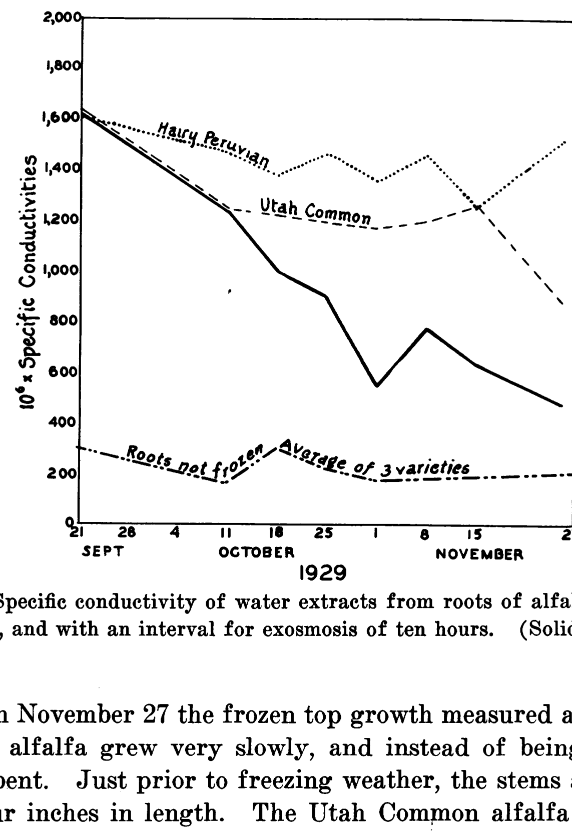

FIG. 1. Specific conductivity of water extracts from roots of alfalfa after freezing for four hours, and with an interval for exosmosis of ten hours. (Solid curve represents Grimm).

right, and on November 27 the frozen top growth measured about 14 inches. The Grimm alfalfa grew very slowly, and instead of being upright, was semi-decumbent. Just prior to freezing weather, the stems averaged about three or four inches in length. The Utah Common alfalfa was somewhat intermediate between the Grimm and the Peruvian with respect to its top growth. This description of the autumnal development of the three varieties is but another way of expressing the differences in dormancy.

No killing frost occurred until late in the season, and the top growth of the Peruvian was not killed until November 19, while the ground remained unfrozen until November 21. On November 27, when the last samples were dug for the conductivity experiments, the ground was frozen to a depth of about five inches. The exosmosis of all such samples tested without further freezing showed no injury from this frozen condition of the soil, while additional artificial freezing for four and fifteen hours (figures 1 and 2) caused considerable damage to the roots. The outward

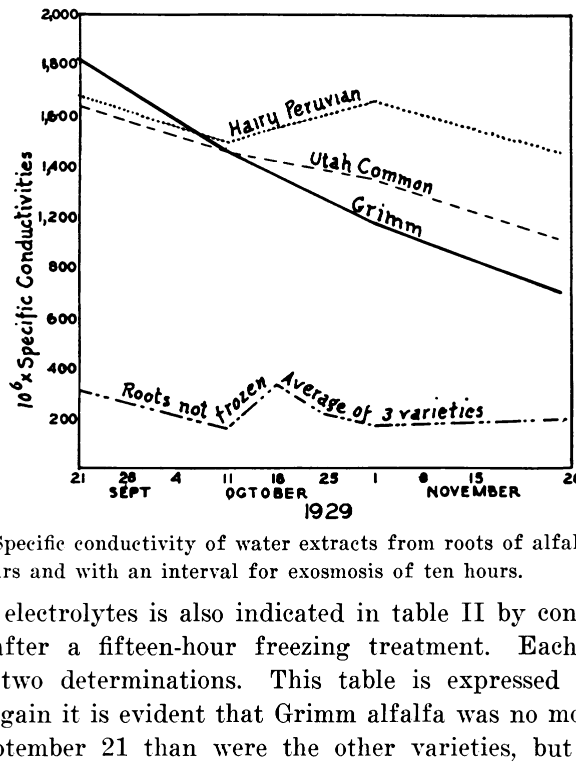

FIG. 2. Specific conductivity of water extracts from roots of alfalfa after freezing for fifteen hours and with an interval for exosmosis of ten hours.

diffusion of electrolytes is also indicated in table II by conductivity measurements after a fifteen-hour freezing treatment. Each value is the average of two determinations. This table is expressed graphically in figure 2. Again it is evident that Grimm alfalfa was no more resistant to cold on September 21 than were the other varieties, but in November, prominent differences are evident.

TABLE II

SPECIFIC CONDUCTIVITIES ($\times 10^6$) IN RECIPROCAL OHMS OF WATER EXTRACTS FROM ROOTS OF ALFALFA FROZEN FIFTEEN HOURS. AN INTERNAL OF TEN HOURS WAS ALLOWED FOR EXOSMOSIS

VARIETY	SEPT. 21	OCT. 11	NOV. 1	NOV. 27
Grimm	1820	1457	1191	696
Utah Common	1647	1463	1357	902
Hairy Peruvian	1680	1499	1689	1572

In order to check the method against another well established variation in hardiness, an experiment was carried out on November 8 and November 15 with alfalfa plants dug from the half of each plat of alfalfa cut four times, so as to facilitate a comparison with the plants cut only twice. With each variety, and in every case, without exception, the roots of alfalfa with low organic food reserves, due to four cuttings, gave a higher value for the specific conductivity than the corresponding roots with high reserves, from plants cut only twice. It has been shown that frequent cuttings lessen the hardiness of alfalfa and such results are corroborated by the conductivity measurements. Table III gives the averages of the two pairs of determinations, which checked very closely, and which were made on roots dug on November 8 and November 15. Differences between Grimm alfalfa with high and low reserves are pronounced, as are also the differences in the Hairy Peruvian with reference to reserves. The Utah Common did not show such wide differences in this respect. In accordance with the results of other investigators, the roots of the several varieties as sampled

TABLE III

AVERAGE SPECIFIC CONDUCTIVITIES OF THE EXTRACTS FROM ALFALFA ROOTS HIGH AND LOW IN RESERVE FOODS. PLANTS WERE DUG ON NOVEMBER 8 AND 15, AND THE ROOTS WERE GIVEN A FOUR-HOUR FREEZING TREATMENT. AN INTERVAL OF TEN HOURS WAS ALLOWED FOR EXOSMOSIS

Variety	Cutting treatments	
	Two cuttings	Four cuttings
Grimm	705	1145
Utah Common	1243	1346
Hairy Peruvian	1302	2093

after the four-cutting treatment showed a significantly lower percentage of dry matter than those receiving the two cutting treatment. In percentage dry matter as well as in conductivity, the Utah Common, however, showed distinctly less difference between the roots with high and low reserves than the other varieties.

As another means of distinguishing differences in the concentration of the exudates in the exosmosis test, the dipping refractometer was used, and was found very useful in detecting the larger variations. The instrument is not as sensitive as the conductivity apparatus and the slight turbidity of the solution often made the readings uncertain when small differences might have existed. No attempts were made to clear the solutions before examining them. The turbidities of these solutions, however, gave evidence of the degree to which exosmosis had proceeded, and very commonly the greater turbidity of the solution from the Peruvian distinguished it from the Utah Common, which in turn was more turbid than that of the Grimm variety. Preliminary experiments indicate that the percentage of solids in the solutions as determined by simple evaporation may be definitely correlated with the conductivity figures. Hydrogen-ion concentration, determined on only one of the experimental dates, October 18, gave no definite correlation with exosmosis and freezing injury. The volume of the precipitate with lead acetate appeared, superficially to be about the same for all varieties.

The concentration of sugars in the solution was also followed throughout most of the season, but no fixed relationship appeared to hold. In general, the Utah Common showed greater exosmosis of sugars than the others, and Grimm showed the least.

In order to correlate the amount of injury from each freezing treatment with subsequent growth, twenty-four plants of each variety were transplanted in soil under greenhouse conditions. In general, the development of these plants gave definite indications of a distinct correlation between high conductivity and freezing injury. It is difficult, however, to express the degree of growth response in specific terms. While the growth recovery after freezing was greatest with the Grimm and always the least with the Peruvian, there were wide variations in different plants of the same variety. Thus, some roots were severely injured at the lower end, but the crown survived sufficiently to send out shoots, while others were injured at the crown and entirely failed to give growth, although the bulk of the root appeared to be relatively sound. Still other roots failed to regenerate even though they seemed to be very slightly injured. The renewal of growth by the plants subjected to the freezing treatments is often very slow, especially when they have been severely injured. In some cases plants were rejected as dead after a period of several weeks, whereas growth finally began after further opportunity. Roots usually die, however, if growth does not occur within three or four weeks, and late growth is always sparse. It is, therefore, difficult at this time to present concise data on the regeneration of growth in the greenhouse, after the freezing treatment. In every case the Peruvian, frozen one and one-half hours, was markedly slower in its early growth recovery than the Utah Common and the Grimm. In no case, did plants other than Grimm survive the four-hour freeze. It seems likely that the maturity of the buds at the crown may be an important factor in regeneration after freezing.

As a further aid in the determination of relative hardiness by the principle of exosmosis from injured tissue, the following colorimetric tests have been tentatively tried on some series, with very promising results. It must be emphasized that the values of determinations of a given ion need not necessarily be in the same ratio as the conductivity values. The organic materials as well may interfere with the tests. However, in this experiment, tests for the chloride ion appeared to correlate very well with the conductivity measurements, as far as they have been carried out. Such tests may well supplant the conductivity measurement in some cases, and in others, they may supplement it.

To compare the concentrations of the chloride ion in the water extract of the roots after freezing, 10 cc. of each solution was treated with 1 cc. of 5 per cent. potassium chromate solution and 15 drops of N/100 silver nitrate. In the absence of chlorides, the solution turns a deep red, or in other terms, presence of chlorides in the extract inhibits the development of the red color of silver chromate. If there is sufficient chloride to precipitate all of the silver, the original yellow color will remain. Solutions from roots of Hairy Peruvian alfalfa, frozen 4 or 15 hours, show much less coloration of red silver chromate than solutions from roots of Utah Common, and in turn, Utah Common show less than Grimm. Thus, Peruvian has given more chloride to the solution, by exosmosis, than the other varieties. The test when applied to the extract from unfrozen roots shows practically negligible exudation of chlorides, and the solution turns a deep red on the addition of the silver nitrate. The colors produced in the solutions remain unchanged for several hours and may be referred to a series of standard chloride solutions, similarly treated for quantitative comparison from week to week.

Organic substances interfered with the tests for nitrates with ferrous sulphate and sulphuric acid, but the colorations produced in 50 per cent. sulphuric acid appeared to correlate fairly well with the conductivity measurements.

These methods should be further standardized. Tests made for the calcium and sulphate ions were not sufficiently definite to appear of value and will not be described here.

A considerable number of possible variables have been investigated in this preliminary work. The size of the roots appears to make no difference in either cold injury or exosmosis, within the limits of our field conditions. Small roots may be cut longer than usual, or thick roots may be cut to one-third of ordinary length to make up the twenty grams of tissue. It does not appear to be necessary to use a given number of roots, so long as a given weight is employed. The roots may be cut off below the crown without affecting the experiment appreciably, but if the crown tissue is cut away, such roots are not suitable for greenhouse tests of growth recovery. Total ash determinations on the roots throughout the fall do not show marked differences in the three varieties. The influence of soil added to the solution in considerable quantities was determined, and does not appear to be one of the larger variables. The rate of thawing may have considerable influence on the amount of injury and the amount of diffusion, but the influence of this variable was not measured. The rate of thawing, however, was quite uniform in all the trials reported in this paper.

The common precautions in conductivity work were employed. The tubes for exosmosis and their stoppers were soaked in distilled water for a day or two before use, and contamination by electrolytes was carefully avoided. With ordinarily careful technique, it does not appear that the unavoidable errors in the conduct of the measurements described are sufficient to affect, in a large degree, the accuracy of these determinations.

There are many incompleted problems left for further study, which will occur to the reader. The possible applications of the method to plants other than alfalfa is obvious. The writers plan to investigate some of these during the coming months, and to further establish the relationships that other variables may have in the reliability of the methods as outlined. It seems very likely that modifications, particularly of the colorimetric tests will be desirable, at least, in applying them to other plants.

Summary

A series of experiments and data has been presented indicating that the degree of resistance of plants to injury by cold weather may be measured by the diffusion of electrolytes and other substances from chilled or frozen tissues into water after such tissues have thawed. The amount of diffusion has been determined with alfalfa roots by conductivity measurements, which have been supplemented by colorimetric tests for chlorides. Within the limits of this investigation there exist correlations between known hardiness of alfalfa roots and the degree of retention of electrolytes by the tissue after freezing.

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