EFFECT OF THE HARDENING PROCESS ON THE WATER CONTENT AND ANATOMY OF SOME HERBACEOUS PLANTS

Thesis for the Degree of M.S.

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THESIS

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THESIS

Introduction.

The annual loss that results from the injury of cultivated plants by low temperatures is great, the effect being spread over a wide range of territory. In localities where the minimum temperature is seldom below zero the injury is frequently as severe as in the more northern areas. This makes the problem of ascertaining the cause of the killing of plant tissue by cold one of general interest and importance. In these investigations a study was made of the relation of anatomy and bound water content to the condition termed hardiness.

Historical.

Theories regarding cause of Death from freezing.

There have been many theories advanced to account for the death of plants resulting from low temperature. The first of these was the opinion of Duhamel and Buffon (15), that death was due to the crushing of the cell wall by ice formation. This was disproved by Goeppert (16), Sachs (48), Nageli (38), Wiegand (56), and others, as will be shown later.

Müller-Thurgau (36) held that the cause of death was water withdrawal from the cell and ice formation usually in the intercellular spaces. Wiegand's work (56) supported this theory. In the light of more recent investigations this must be broadly interpreted to include such secondary effects as would in some way change the character of the cell contents. Molisch (35) agreed with this broader interpretation of Müller-Thurgau's hypothesis.

Gorke (19) advanced the theory that death from freezing is due to "salting out" of proteins. The removal of water from the cell causes increased concentration of the cell sap, and proteins are precipitated much as from strong salt solutions in vitro. Schaffnit's results (50) supported this explanation.

Gorke (19) noted increased acidity in the sap upon freezing and thought this might be a factor in protein precipitation.

Harvey (25) noted Gorke's observation; accumulated considerable data, and concluded that increased acidity is the cause of protein precipitation.

Rosa (47) considered pentosans (hot water soluble) to be an important factor in protecting plant cells and tissues from water loss. Hooker (27) and Newton (39) give evidence in support of this theory.

Chandler (11) writes: "The theory that the disorganization of the protoplasm is a mechanical injury caused by the pressure of the ice mass seems to explain more of the experimental results with the freezing of plants than does any of the others". He admits, however, that the proof of this is not conclusive.

Evidence on Water Loss and Ice Formation.

Many investigators have observed water withdrawal from the cell and subsequent ice formation.

Müller-Thurgau (36) pointed out that the loss of water from the cell when ice formation commences is very rapid at first, becoming gradually slower. It ceases when there is equilibrium between the force of crystallization and imbibitional forces plus osmotic pressure within the cell. For apple tissue he gave the following water losses at stated temperatures:-

Temperature.	% Total water frozen out.
-4.5°C.	63.8
-8.6°C.	72.4
-15.2°C.	79.3

Müller-Thurgau also found that ice formation usually occurs in the intercellular spaces. Ice did not begin to form at all points simultaneously, but water moves from adjoining or nearby spaces to points at which ice formation commences. Even when ice mass becomes so large that the cells are torn away from each other, they may not be killed. Sachs (48) and Molisch (35) noted ice formation as chiefly confined to the intercellular spaces, although it occurs within the cell when the temperature fall is very rapid.

Wiegand (56) agreed with Müller-Thurgau. He observed that ice forms in broad prismatic crystals perpendicular to the excreting surface. At -18°C. to -25°C. the cells near the ice mass are, in certain cases, in a state of collapse but upon thawing re-absorb the water and resume their normal condition. He reported ice formation in twigs and buds of woody species in winter as taking place chiefly within the intercellular spaces. Ice is at times found within the cell. The spaces between the bud scales were packed with ice crystals. Dorsey (14) also reported this condition in plum fruit buds, noting that there is no ice in the space above and around the floral parts, between them and the bud scales.

Adams (1) working with moist seed observed conditions of water withdrawal and re-absorption similar to those previously mentioned. He stated, as did Wiegand, that if the tissue is killed no re-absorption of water takes place.

Greeley (23) found that water is extruded from Spirogyra cells at 1 to 2^0 above freezing and a condition similar to plasmolysis is brought about. Morphological changes similar to plasmolysis are produced at will by lowering the temperature.

Wiegand (57) advanced two theories to account for cellular water loss on freezing. These were: (1) Extrusion (2) Attraction.

The first theory is based on the supposition that the cell gives up water at low temperatures by contraction and squeezing. This condition may be due to change of permeability as Osterhout (42) has shown that freezing, among other agencies, greatly changes cellular permeability. Wiegand favors his "attraction theory" as the more probable explanation. He considers a film of pure water to be adhering to the outer cell wall, around the intercellular spaces. The cell wall and cytoplasm contain water of imbibition while the vacuole contains a water solution of various substances. The outer film of water freezes first. The whole system was in a state of equilibrium and to restore this water is drawn from the cell wall. The latter draws on the cytoplasm and the cytoplasm takes from the vacuole. Water exchange goes on until the force of crystallization and the forces within the cell are again in equilibrium. No more water freezes out until the temperature is lowered. The forces holding water in the cell become stronger as the amount of water is reduced and less water is frozen out at each degree of lowering of temperature. This agrees closely with Müller-Thurgau's findings with apple tissue. Wiegand also states that the forces withholding water from freezing are dependent on the water content, and inversely proportional to it. In plants with high water content the surface film is thicker and therefore, more weakly held against freezing. He thought that, in succulent tissues, a large part of this water is withdrawn at the initial freezing.

Müller-Thurgau (36) and Voigtlander (55) cooled tissue well below the temperature at which it killed. When the tissue was warmed back without ice formation, no damage occurred. Wright and Taylor (59) proved this to be true with potatoes, and reported that where ice formation occurs before warming, typical frost injury appears. With woody plants ice formation would occur well above the death point.

Chandler (10) stated that leaves of tender plants whose surfaces are wet, kill much more severely than those which are dry. Harvey (25) demonstrated that cabbage leaves cooled below the freezing point may be caused to freeze internally in spots by droplets of water on the surface freezing and inoculating the tissue directly beneath them. Injury is greatest at the point on the leaf where crystallization commences. This agrees closely with certain findings of Müller-Thurgau and Wiegand given previously. Harvey (26) also demonstrated with Cinereria, Echiveria and other plants the importance of waxy or hairy coverings in preventing the inoculation mentioned above. Undercooling to -5 to -6°C. can be attained without injury, when these coverings are not removed.

Ice Pressure Effects.

It will be recalled that under "ice formation" mention was made of the work of Wiegand, Müller-Thurgau and others, reporting the collapse and pressing out of shape of cells or groups of cells by ice pressure. Cells might be torn from each other and still return to normal on thawing, if death had not ensued.

Chambers (9) stated that the cytoplasm of the egg of some marine animals coagulates with ease on mechanical injury. Compression often causes the whole egg to coagulate in a solid mass, although continuous gradual pressure is much less harmful than short, rapid application. Injury is accompanied by swelling and an increase in acidity of the part involved.

Bancroft (3) quotes Ehrenberg's opinion that the coagulation of colloidal solutions on freezing is due to the pressure of the expanding ice.

Keith (29) carried on work with Bacillus coli held at low temperatures in water. At -20oC. most of them are killed in five days and all are dead in a few weeks. If during freezing the ice is kept worked into a loose mass, a large percentage of the bacteria remain alive for many months at -20°C. Many survive over a long period when 40 and 45% glycerin is added to the water.

Dorsey and Strausbaugh (14) studying flower buds of the plum that had been killed by freezing found that the plasma membrane of the cell shows as a dense spreading smudge of stain. In the case of sections similiarly treated, but killed by laboratory reagents, a distinct limiting membrane is observed. In many of the cells killed by freezing details of the nuclear organization are completely obliterated. The authors state, "It is apparent, that the killing of the embryonic buds by low temperature brings about fundamental changes in the cell organization, particularly in the nucleus".

Differences between Hardy and Tender Tissues.

Considerable data are available dealing with differences between tissues of varying degrees of resistance to low temperature, either measured or stated from observation. These differences may be of water content, rate of water loss, sap density, carbohydrate changes, etc.

Water Content and Dry Matter of Tissues.

Bakke (2) gives the following percentage of water (average of 17 varieties) found in apple twigs at various periods.

Period.	% Water
Dormant	45.765
Bud Swelling	52.56
Blossoming	64.19
Summer Growth	58.92
Wood Ripening	52.55

Chandler (10) could find but slight differences in the water content of the cortex of Elberta peach and Jonathan apple stems from November to May, nor were the differences consistent.

Johnson (28) reports a marked seasonal increase in water content of peach buds in Maryland, the increase being parallel with the increase in tenderness of the buds toward spring. Greensboro, a hardy variety, has a lower percentage moisture than Elberta, a more tender variety.

Chandler (10), Potter (46) and Carrick (8) report that apple roots which are allowed to absorb moisture suffer somewhat more injury by cold than unsoaked roots and much more than roots which are dried.

Kiesselbach and Ratcliff (30) found freezing to death of seed corn is directly proportional to the moisture content and duration of exposure. They also noted that the temperature at which ice formation commences depends largely on moisture content. With 60 to 80% water, ice forms just below 32°F., while with 18% moisture in air dry seed, no ice is formed at -10°F.

Rosa (47) found a greater percentage of dry matter in hardy than in tender plants. The differences do not parallel the killing temperatures, however, and are small in many cases. The following table compiled from his data for cabbage plants shows this.

TABLE I.

Treatment	% dry matter	Relative hardiness.
Greenhouse plants not hardened	9.38 10.52	Killed at -4°C. in 2 hrs.
Minimum moisture. greenhouse	9.22 10.9 12.8	Uninjured at -4°C. in 2½hrs. " " -3°C. in 2 hrs. " " -5°C. in 1 hr.
In cold frame 3 wks.	13.25	Slightly injured at -6°C. in 2 hrs.
Compost M/10 $NaNO_3$	9.31	Slightly injured at -6°C. in 30 min.
Sand M/10 $NaNO_3$	6.52	(Killed at -6°C. in 30 min. (Not injured at -3°C. in 30 min.

It would seem that in the last two cases shown in Table I. the percentage of dry matter is not the important factor.

Schaffnit (50) reports the amounts of dry matter in wheat varieties in direct proportion to their known hardiness. Gorke (19) states that more hardy plants have a greater percentage of dry matter.

Many other quotations might be given showing increased dry matter content for hardy varieties. It would appear that high percentage of dry matter may be correlated with hardiness but other factors may have as great or greater importance in enabling a plant to withstand low temperature. Most of the data deal with different varieties and not the same variety in a hardened and tender condition. May it not be that water content and not dry matter is the important factor? Thus the amount of water may increase in woody plants as they become more tender but it does not necessarily follow that the actual amount of dry matter changes, altho it would decrease when measured as percentage of green weight or ratio of water to total solids.

Chandler (10) concluded after experimenting with a number of tender plants and fruits of the peach, when in both a turgid and a wilted condition, that wilting did not increase cold resistance, except in occasional instances. Slow loss of water in dormant peach buds increased resistance to low temperature. It is pointed out, however, that this is entirely different from rapid wilting, in that the slow drying permits of changes other than a decrease in the amount of water. However, it is problematical if any changes would occur at the low temperature prevailing.

Beach and Allen (4) found that drying apple twigs before freezing lessened the injury. Salmon and Fleming (49) working with greenhouse grown cereals found slightly less injury in wilted than turgid plants.

Irmscher (58) showed that drying any of the species of moss he used increases their resistance to cold.

Wiegand (57) considered that the greater the water content of plant tissue, the thicker would be the film of water on the surfaces of the imbibing substance. Therefore, the force by which the outer layers of this film are held would be weaker, hence more easily withdrawn to form ice.

<u>Rate of Water Loss.</u>

Beach and Allen (4) found that in general the wood of the hardiest varieties of apples was more resistant to water loss than more tender varieties.

Strausbaugh (51) reported that semi-hardy varieties of plums have a fluctuating moisture content in twigs and buds, while the water retaining power of hardy varieties is constant. During warm periods, (practically never above $0^{o}C.$) the buds of semi-hardy varieties increase in water content while at a lowering of temperature the process is reversed with equal rapidity. Buds of hardy varieties have a nearly constant water content. This was further demonstrated by drying twigs with buds attached, over sulphuric acid of various concentrations. The tests covered a period from November to March.

Boswell's (5) work shows a faster rate of dehydration for tender than for hardy plants. Hardy tomatoes lose water at a slightly more rapid rate than tender cabbage.

<u>Freezing Point Depression of Sap.</u>

Lewis and Tuttle (31) examined leaves of Picea canadensis, Linnaea, Pyrola and bark of Populus. There is but slight differences in sap concentration from October to April, the maximum occurring in March after cold weather is past. Harris and Popenoe (24) found that the hardiest species of avocado have the lowest freezing point depression.

Ohweiler (43) investigating the results of a late Spring frost at the Missouri Botanic Gardens, concluded that in the majority of cases plants with the greatest sap concentration suffered least, altho there were many exceptions.

Newton (41) could find no relation between the freezing point depression and resistance to cold of wheat varieties. Salmon and Fleming (49) came to similar conclusions using oats and wheat.

Rosa (47) gives data on this subject similar to that hereafter quoted from Chandler. His differences are quite large but the lowest freezing point of any of his samples of cabbage or tomatoes is that for medium and hardy lots of tomatoes. These killed at $-2^{o}C.$, a higher temperature than any of the cabbage and only slightly different from tender tomatoes. Differences within a lot of similarly treated plants were frequently as great or greater than differences between different lots.

Chandler (10) and Harvey (25) found a lower freezing point for expressed sap of hardy than for tender plants. Table II. taken from Chandler shows some of the variations of freezing point of sap in relation to temperature injury. He subjected plants for

24 hours with roots in solutions of strengths measured by the freezing point noted.

TABLE II.

(Chandler (10) pp. 167.)

Treatment.	Temp. °C.	Leaves. % killed	% killed & partially killed	Δ
Tomato.				
Glucose (.460)	-3	63.0	67.6	.765
Cane Sugar (.435)	-3	83.3	88.3	.925
Glycerine (.430)	-3	72.8	88.5	1.070
Water	-3	100.0	100.0	.700
Cabbage				
Glucose (.44)	-4	100.0	100.0	1.190
Cane Sugar (.77)	-4	100.0	100.0	1.230
Glycerine (.66)	-4	66.6	66.6	1.270
Water	-4	66.6	66.6	1.080
Lettuce.				
Cane Sugar (.677)	-3	0.	25.0	.652
Glycerine (2.820)	-3	00.	35.0	.728
Water	-3	20.0	65.0	.597

There is considerable difference in injury to plants from the same solution but subjected to different temperatures. However the extract given in Table II. is fairly representative. From Table II. one would conclude that altho the freezing point may vary directly as the temperature required to kill, yet this relation is incidental. Other factors than those which make for hardiness, have a greater influence in some instances on the depression of the freezing point.

Wiegand (57) considered sap concentration of slight importance and then only at the inception of freezing.

Rosa (47) states "It may be said that the apparent increase in osmotic concentration of the sap in plants on hardening is merely co-incidental".

The findings of Carrick (7) are rather indicative, especially from the standpoint of studies with expressed sap. He reports that the freezing point of expressed apple-fruit juice, measured with the Beckmann apparatus, was significantly higher than the freezing point of the cell sap as determined within the normal unfrozen apple by thermo-junctions.

Acitity, Carbohydrate and Protein changes on hardening.

Gorke (19) suggested that sap concentration in freezing acted on the protoplasm and precipitated the proteins. He reported proteins precipitated from Begonia sap at -3^{0} while -40^{0} was required to precipitate them from pine needles. He also noted increased acidity on freezing expressed sap. Schaffnit (50) in his experiments with rye could not precipitate protein from hardy plants.

at the higher temperature which will precipitate proteins from the sap of tender plants. Furthermore he could prevent proteins being precipitated from the expressed juice by the addition of small quantities of sugar. The conclusions were that starch in hardy plants changes to sugar on the approach of winter, and protects the protoplasm from changes similar to protein precipitation.

Pojarkova (45) dealt with 14 Ribes, 10 Lonicera, 11 Acer also Berberis, Amelanchier, and Corylus. Those species in which a large proportion of starch is transformed into sugar at the approach of winter manifest profound dormancy. The relation of the instensity of winter dormancy to cold resistance, as reported elsewhere for plums, is shown only in Ribes. Relatively cold resistant species have a profound dormancy.

Lidforss (32) found with evergreen plants in South Sweden that most of the starch is changed to sugar in cold weather. Starch again appears on the return of warm weather.

Müller-Thurgau (36) Michael Durand (34) and other investigators have noted the accumulation of sugars in plants on the approach of winter.

Dorsey and Strausbaugh (14) found chiefly protein substances, fat and dextrin in plum fruit buds in winter.

Rosa (47) showed that total and reducing sugars increase in hardened plants, being greater in cold frame hardened than those hardened by other means. The increase in sugars is greater in cabbage and lettuce than in the tomato. He says, "There is no direct evidence that the absolute quantity of sugars present in a plant is directly related to its cold resistance". In lettuce, cauliflower and cabbage the amount of total polysaccharides is slightly less in hardened than tender plants, the decrease being due to the reduction in starch. With tomatoes they show a large increase in the hardened plants due to starch accumulation. Rosa points out this larger accumulation of starch as an indication of differences between plants which can be hardened and those which cannot. He stated, however, "It appears probable that an increased sugar content in hardened plants is more likely one of the manifestations of the condition of being hardy than a direct cause of cold resistance". Rosa also found increased pentosan content (hot water soluble), in plants which had been hardened but his analytical methods, as he stated later, were unsatisfactory, though considered the best at that time.

Hooker (27) and Newton (39), using Rosa's method of analysis, found pentosan content to increase in the hardier parts of apple wood and in more cold resistant varieties of wheat.

Harvey (25) in his work with cabbage, stated that the acidity increases greatly on freezing. He precipitated a much greater percentage of protein from the expressed sap of tender than of hardy cabbage, both by freezing at -4°C. for two hours and treating with sulphuric acid sufficient to increase the acidity equal to the increase caused by freezing at -3°C. He concluded that the increased acidity causes protein precipitation and death. Harvey also found an increase in the amino nitrogen in hardened plants and considered this a significant result of the hardening process, indicating a reason for non-precipitation.

Maximow (33) froze thin sections from the upper side of red cabbage leaves and Tradescantia in pure water and solutions of various substances. The sections were placed in the solutions and immediately frozen, as it was found this gave as much protection as when they were soaked in the materials previous to freezing. Some of his results are given in the following table taken from Chandler (11).

TABLE III.

Freezing Temperatures of Sections of Cabbage Leaves.

Material in which frozen	Minimum temp. withstood.
Pure water	-5.8^{o}C.
Glycerine 2N conc	-22^{o}C.
Glucose 2N "	-32^{o}C.
Methyl Alcohol 2N conc.	-11.1 to-17.3^{o}C.
NaCl 2N conc.	-32^{o}C.
$CaCl_2$ 2N conc.	-22.0 to -32^{o}C.
$NaNO_3$ 1N "	-17.3^{o}C.
KNO_3 1N "	-7.8^{o}C.
Sodium Oxalate 1N conc.	-5.8 to -7.8^{o}C.
Potassium " 1N "	-7.8 to -11.1^{o}C.
Expressed Red Cabbage Sap	-11.1 to-17.3^{o}C.
" and Boiled Red Cabbage Sap	-11.1 to-17.3^{o}C.

The figures are interesting because of the fact that some substances which would kill the tissue at temperatures above freezing gave protection equal to that afforded by glucose and other sugars not shown. Both Schaffnit (50) and Lidforss (32) considered that sugars protect against salting out of proteins. The sap of the cabbage exhibited a distinct protective action which does not altogether agree with findings of Harvey (25), for the acidity must have been high. Toxic action is apparently greatly reduced by low temperature.

Vass (54) found a protective action exhibited by dextrose and glycerine bacteria. He concluded, as did Maximow, that the protective action was due to the keeping of a film of unfrozen water in contact with the outer layer of the protoplasm, not therefore to any direct effect on the protoplasm or cell contents.

Objects of Experiments.

The aim of this work was to ascertain the effect of the hardening process on the hydrophyllic colloid content of the sap and gross anatomy of certain plants. The freezing point of expressed sap, rate of tissue dehydration, percentage of solids and water in sap and tissue were studied in their relation to killing temperature. The use throughout of plants of like variety and condition with controlled methods of finding cold resistance render the investigations more valuable. The object throughout was to add something to the available information on cold resistance in plants.

Materials.

Cabbage was used to represent plants which could be hardened to withstand a fairly low temperature. The tomato, the other plant chosen, can be grown so that it is to all appearances very hardy, yet it will survive temperatures but little below $0^{o}C$.

The work here reported was carried on during the summers of 1925 and 1926. The plants used were uniform in vigor and size before undergoing treatment and were of the same age. All were grown in outdoor cold frames, covered only when protection from rain was necessary.

1925 Experiments.

Series A.

A good loamy soil, except lot 4, reaction rendered distinctly alkaline with $Ca(OH)_2$.

Lot 1. Plants watered freely. Tender

Lot 2. Watered only enough to prevent wilting. Well hardened.

Lot 4. The soil was pure sand to which had been added a very little of that used for other lots in this series. Plants watered freely.

Lot 5. Ten days before being used plants were taken from Lot 1. and given an application of $NaNO_3$, one gram in 20 c.c. water to each 4" pot. This was repeated in five days.. The result of the first application was evident in two days and plants at the time tested were succulent in appearance.

Series B.

Soil similar to A. except that it gave a slightly acid reaction. Soil test pH 6.2 to 6.7. (soiltex).

Lot 1. Plants watered freely. Tender.

Lot 2. Watered only enough to prevent wilting.

1926 Experiment.

All plants were grown in soil very similar to that used in 1925, being nearly neutral in reaction. Five inch pots were used, and both cabbage and tomatoes treated as follows.

Lot 1. Plants watered freely.

Lot 2. Hardened by withholding water.

Lot 3. Pot bound. Water supplied freely.

Methods.

Estimation of Killing Temperature.

Tests of killing temperature were carried on in a four-chambered "Frigidaire" freezer. It was very easy with this machine to lower the temperature gradually and steadily.

At the start the temperature was at $0^{o}C$. or just below. Four pots were put in each compartment. The temperature rose at once to around $5^{o}C$. due to the heat from the plants, soil and pots. In from $1\frac{1}{2}$ to 2 hours it was down to the starting point. In 1926 the temperature was lowered $1\frac{1}{2}$ to $2^{o}C$. every hour. It took therefore approximately four hours to reach $-3\frac{1}{2}^{o}C$. at which point all point all the tomatoes had succumbed. The cabbage plants were frozen slightly faster, as their minimum temperature of -7°C. was reached in five hours. The temperature in 1925 was lowered more slowly. It does not seem, however, that even the former lowerings would be considered excessively rapid freezing. Possibly the differences in killing temperatures would have been greater had the fall been less rapid. Chandler (10) points out the great injury even to dormant buds and twigs by a very rapid lowering in temperature as compared with slower fall. He lowered the temperature to -20°C. in from 1 to $1\frac{3}{4}$ hours with rapid freezing, while in slow freezing 7 to 10 hours were required to reach that temperature.

Readings were taken every 30 minutes, plants being removed from the freezer at each reading, starting when the leaves first became stiff. They were exposed for observation of injury at room temperature, 21 to 27°C., out of direct sunlight. Half an hour was usually sufficient to bring out any injury.

A part of this work consisted in a study of the anatomy of the plants used in 1926. The object was to determine such general differences as might exist between different treatments and correlate them with cold resistance. The stem between the second and third nodes, leaf petiole, and middle part of leaf blade were selected for sectioning. The parrafin method was used throughout.

Methods of Sap Study.

The plants in each lot were cut off at the surface of the ground, weighed, cut into fine pieces with a knife and thoroughly mixed. A fifty gram sample was taken for rate of dehydration determinations. Another portion was put in a tightly corked bottle, and suspended in an ice and salt mixture, at -18°C. The container with the freezing mixture was insulated from room temperature with hair felt between it and an outer vessel. All samples were taken in the late afternoon and left in freezing mixture until the following morning. The bottles were then removed, washed clean, dried and the contents thawed by placing the bottle in warm water. No moisture was lost from the tissue before pressing, altho it was wet and soggy. After thawing the material was packed in a clean steel cylinder and immediately pressed in an hydraulic press. Pressure was applied only fast enough to keep the sap dripping, this operation taking from 15 to 25 minutes. The 1926 samples were wrapped before placing in the cylinder in unbleached muslin, which had been boiled several hours in changes of distilled water and thoroughly dried. In 1925 a pressure of four tons, and in 1926 one of 16 tons to the square inch was used. The residue in each case was dry to the touch.

The expressed sap was at once centrifuged for ten minutes at 1500 r.p.m. to remove debris. The liquid remaining was kept at a temperature close to 0°C. until used.

The remainder of the procedure and apparatus used for estimating hydrophilic colloids was as used by Crist (12) pp. 15, 16, following the methods of Gortner and his co-workers, (20), (21), (22), (40).

The samples of tissue for drying were put in 600 c.c. beakers in an electric oven held at 90°C. Weights were taken at stated intervals.

TABLE IV.

Killing Temperature, Freezing Point Depression of sap and Percentage Water in the Sap and Tissue of Cabbage and Tomatoes.

Treatment	Killing Temp.	Δ	% water in Expressed sap	% water in Oven dried tissue
CABBAGE. 1925	°C.			
Watered freely				
Alkaline soil	1 hr. at -5to-5½	.747	93.6	88.2
Slightly acid soil	1 " " -5½	.815	93.2	86.8
Watered sparingly				
Alkaline soil	½ hr. at -6to-7	.834	94.1	90.0
Slightly acid soil	1 " " -4½to-6½	.883	94.1	90.0
Poor soil	1½ hr. at -6	.806	93.4	89.2
Treated with $NaNO_3$	1 " " -4½to-5½	.930	93.5	88.0
1926				
Watered freely	½ hr. at -5	.766	93.7	88.2
Watered sparingly	½ " " -7	.896	93.3	83.0
Pot bound	½ " " -5	.843	92.5	81.2
TOMATOES 1925				
Watered freely				
Alkaline soil	½ hr. at -3to-3½	.610	94.9	88.1
Slightly acid soil	1½ " " -2	.653	94.2	85.7
Watered sparingly				
Alkaline soil	1 hr. at -3½	.838	93.4	90.0
Slightly acid soil	1 " " -3¼	.900	92.1	86.3
Poor soil	½ hr. at -3	.624	95.0	90.0
Treated with $NaNO_3$	1 " " -2½	.780	93.1	86.2
1926				
Watered freely	½ hr. at -3½	.620	94.6	86.0
Watered sparingly	½ " " -3½	.880	93.0	84.2
Pot bound	½ " " -3	.621	94.1	85.4

Presentation of Data.

Killing Temperature and Freezing Point Depression.

Data in Table IV. show that the freezing point may vary directly as the killing temperature but that it does not consistently do so. The differences in the freezing point of expressed sap are larger between lots of tomatoes than lots of cabbage yet the former had nearly equal killing temperatures. The lots treated with sodium nitrate proved the most tender, but the cabbage so treated had the lowest freezing point of any lot. The freezing point was always higher for plants grown on acid than on alkaline soil.

Relation between Killing Temperature and Percentage of Total Water.

Instances can be found in Table IV. in which the percentage of total water drops as the killing temperature lowers. This is, however, the exception with cabbage. Tomatoes show this lowering of water content consistently for lots which would be hardy in plants that can be induced to resist temperature lowering to any degree. However, the tomatoes showed very little differences in killing temperature. There was also as great a variation in water content among lots of hardy plants in different years or on different soils as between lots receiving different treatments.

In the 1925 experiments the "freely watered" cabbage had a lower water content than the "sparingly watered" lots. In 1926 this was reversed. Taking all lots into consideration there is no consistent relation between killing temperature and percentage of total water. Any correlation between the two, then, would have to be considered merely incidental, at least as far as these experiments are concerned.

TABLE V.

Relation Between Killing Temperature, Bound Water, and Percentage Solids in Juice and Tissue.

Treatment.	Killing temp.	Bound Water %total water	% solids in Expressed juice	% solids in Oven dried tissue
CABBAGE 1925	°C.			
Watered freely				
Alkaline soil	1 hr. at -5to-5½	6.46	6.4	11.7
Slightly acid soil	1 " " -5½	6.09	6.8	13.2
Watered sparingly				
Alkaline soil	1 hr. at -6to-7	4.50	5.9	10.0
Slightly acid soil	1 " " -4½to-6½	3.20	5.9	10.0
Poor soil	1½ hr. at -6	2.50	6.6	10.8
Treated with $NaNO_3$	1 " " -4½to-5½	5.10	6.5	12.0
1926				
Watered freely	½ hr. at -5	10.99	6.3	11.8
Watered sparingly	½ " " -7	10.99	6.7	17.0
Pot bound	½ " " -5	3.45	7.5	18.8
TOMATOES 1925				
Watered freely				
Alkaline soil	½ hr. at -3to-3½	7.59	5.1	11.9
Slightly acid soil	1½ " " -2	5.40	5.8	14.3
Watered sparingly				
Alkaline soil	1 hr. at -3½	10.80	6.6	10.0
Slightly acid soil	1 " " -3½	7.20	6.9	13.7
Poor soil	½ hr. at -3		5.0	10.0
Treated with $NaNO_3$	1 " " -2½	6.57	6.9	13.8
1926				
Watered freely	¼ hr. at -3½	7.45	5.4	14.0
Watered Sparingly	½ " " -3½	8.44	7.0	15.8
Pot bound	½ " " -3	6.43	5.9	16.6
# Cauliflower I.	4/5 dead 1hr.-8	6.5	5.9	12.0
Cauliflower II.	dead 1 hr. -8	9.1	6.1	15.0

These plants were originally, I. tender; II hardy, but had become badly pot bound before use.

Bound Water, Killing Temperature and Percentage Solids.

The cabbage and cauliflower have a percentage of "bound water" varying inversely as the killing temperature, with few exceptions. Tomatoes show a greater percentage of "bound" or colloidally held water in "hardened" than tender lots, altho there is little or no difference in killing temperature.

"Pot bound" and "poor soil" lots of cabbage show a very low percentage of bound water, altho the killing temperature is equal to or greater than tender lots or those showing much greater percentages of bound water.

Plants treated with sodium nitrate were tender yet they showed a relatively higher amount of "bound water".

These data show chiefly a negative correlation between "bound water" and killing temperature, altho exceptions occur.

There is little relation between "bound water" and percentage solids in tissue and juice.

The percentage of bound water is low throughout on acid soil, as compared with that of plants on alkaline soil.

Figure 1.

Dehydration of Cabbage Tissue.
50 gm. samples at 95°C.

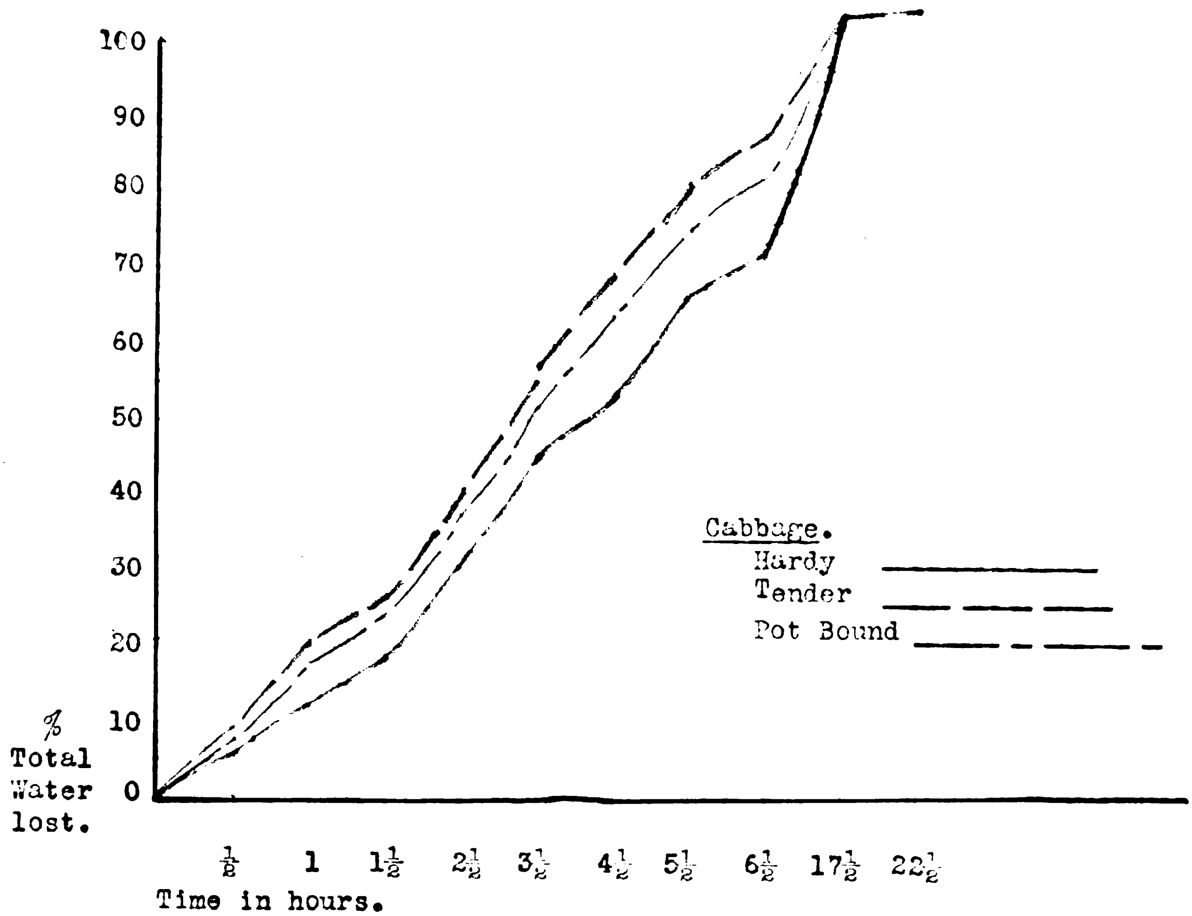


Figure 2.

Dehydration of Tomato Tissue.
50 gm. samples at 95°C.

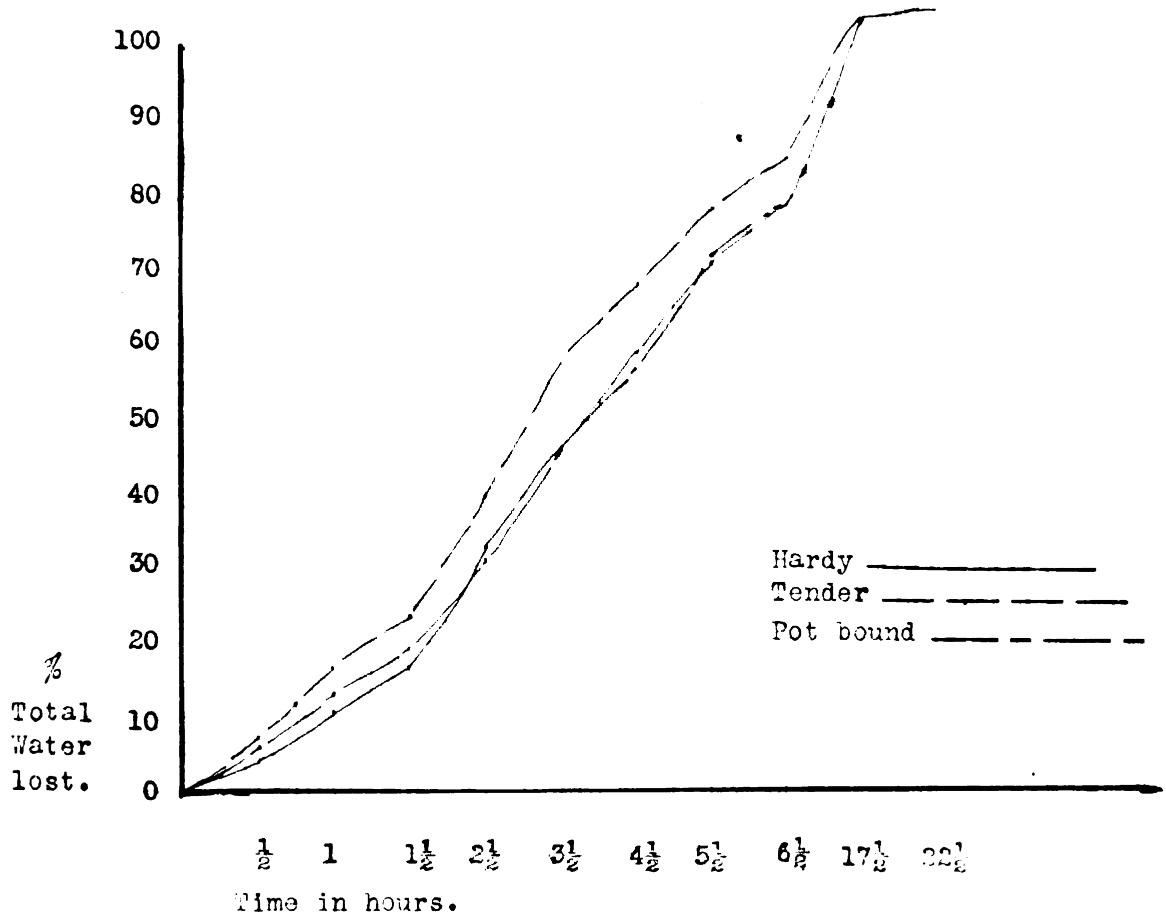
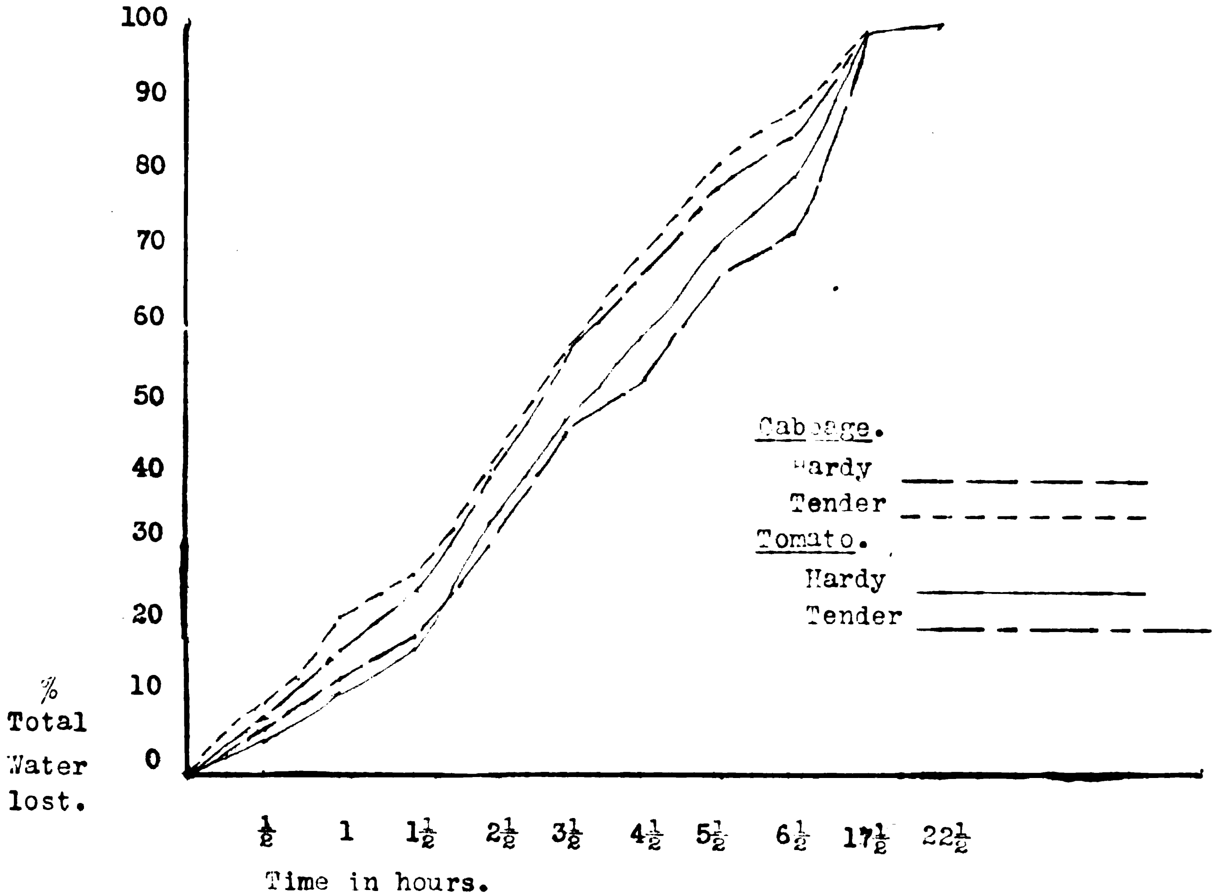


Figure 3.

Dehydration of Cabbage and Tomato Tissue.
50 gm. samples at 95°C.



Relation Between Rate of Dehydration, Bound Water, and Killing Temperature.

The graph in Fig. 1, shows the rate of dehydration of cabbage tissue. Although the variations in all dehydrations, including this, were not large, the order from the greatest to the least was tender, pot bound, hardy. There is no relation to bound water nor any significant relation to killing temperature.

Data similar to the above is given for tomato tissue, in Fig. 2. The results are the same as for cabbage except that the rate of dehydration of pot bound and hardened plants is almost identical.

In Fig. 3. are presented the rates of dehydration for hardy and tender cabbage and tomatoes. The order from the greatest to the least rapid in loss of water is tender cabbage, tender tomato, hardy tomato, hardy cabbage. The latter at first lost water faster than the hardy tomato. There is no definite correlation between rate of dehydration and bound water, or between rate of dehydration and killing temperature.

Anatomy.

In plates 4 to 9, inclusive, are shown one series of cabbage and one of tomato tissue. Other sections are not included because of lack of space. However, these show differences similar to the sections included.

The chief points of difference between the tender and hardy tissues are in size of cell, thickness of cell wall and compactness of cells. These are much the same as differences noted by Bruner and Weaver (6), Pool (44), and others as existing between tissues of xerophytic and mesophytic plants.

The leaf petiole of the hardy cabbage show smaller, much thicker walled cells in the cortex. These are also more closely packed together than is the case in the tender cabbage. In the latter the cortex cells are loosely arranged. The pot bound plants occupy an intermediate position. The cells of the epidermis of the leaf petiole exhibit like conditions but more marked even than in the cortex. The collenchyma directly below the epidermal layer is so small celled in the hardy tissue as to give the appearance of a double or triple layered epidermis. The pot bound plants have even smaller, more closely packed cells in the epidermis and subepidermis than the hardy tissue. .

The cabbage stem cross-sections (not included) show similar and evident differences. These are not quite as marked as in the leaf petiole. The pot bound plants have, in this case, smaller, thicker walled, more closely packed cells than even the hardy lot.

Tomato sections do not differ markedly from the cabbage, in comparison with each other. The fibro vascular system as shown in cross section, appears to be larger in the tender than either of the other two tissues.

In comparing hardy and tender tissues, there are certainly differences which appear to be correlated with the "hardened" condition. These are similar to those noted by other workers as being associated with xerophytic or mesophytic conditions. This would be expected from the treatments the plants received. It is evident from a study of the gross anatomy of these plants, that there are distinct differences which may be associated with cold resistance. These differences are not causes of hardiness nor can they be taken as measures of cold resistance. Pot bound plants, relatively tender, approached the hardy lots very closely in structure, and tomatoes did not differ greatly from cabbage in comparisons between lots.

Discussion.

From the material submitted in the review of literature it is clear that the weight of evidence supports changes caused by water loss from the cell as the reason for death by low temperature. There is considerable evidence pointing to important changes in the protoplasm of the cell resulting from death by freezing.

The salient points of the cold resistance problem, from the water relations standpoint are firstly: Is increased water retaining power, of hardy plants, the important factor or is it the amount of freezable water? For example, in a hardy tissue, which has a higher water retaining power than a more tender one, this hardy tissue can be made more tender by allowing it to imbibe water. Is the original water retaining power changed? If not, this hardy tissue would show a higher percentage of freezable water, but it would be due entirely to the higher water content. Secondly: Is water content always the important factor? Thirdly: Does death result from changes within the cell caused by its lowered water content or are these changes caused by ice pressure from without the cell?

Chandler, Carrick, Potter, Rosa, Salmon and Fleming, Beach and Allen, Irmscher, and others found that with various plants hardiness could be increased by decreasing the water content and the first three, that increasing the water content decreased hardiness. This was in most cases accomplished quickly so that in all probability little change other than that of water content occurred. Chandler did not find this to hold true for many of the tender tissues he used. Other workers have found increased water content correlated with a condition of decreased hardiness, while some investigators have found increased dry matter in hardy plants. As has been pointed out (pp. 8.) this latter might be considered another way of stating decreased water.

The work reported in this paper indicates very little difference in the total water content of hardened and non-hardened tissue and no regular relation between it and the killing temperature. It is clear then that under some conditions the amount of total water is not the important factor in cold resistance. Some condition other than water content made the difference in cold resistance between both similarly treated lots of different kinds of plants and different lots of the same plant.

It has been demonstrated by Strausbaugh, also Beach and Allen, that woody tissues, of certain tender varieties are less retentive of water than corresponding tissues of hardy varieties. Boswell found this to be true with cabbage and tomato tissue, in which case the same variety was hardened to resist cold. The findings of this work do not bear out that point of view except with cabbage. It is significant that rate of dehydration had no relation to the percentage of bound water. This agrees with the work of Crist (12) in which he found that bound water did not parallel the water contend or rate of dehydration.

Newton (41) found that sap was strongly held against pressure in green tissues of wheat plants in late Fall, when such was not the case in early Fall. Other reports from dilatometer experiments.

indicate that by that method more water is held in hardy tissue than tender. In another paper Newton reports hydrophyllic colloid content to be directly proportional to hardiness. The writer's work shows no relation between cold resistance and bound water (hydrophyllic colloid content) as measured by the methods used. It is clear that conditions other than those affecting cold resistance can change the percentage of bound water. Also the hardened condition may give an increase in bound water over succulent tissue without increasing the cold resistance, as the tomato plants show. The conclusion seem justified in view of the data submitted, that where the percentage of bound water varies directly as the hardiness, this relation may be only incidental. It is possible, however, that there may be need for further standardization and perfecting of technique in expressing sap from tissues. Newton, Brown and Martin (60) in a recent paper have shown the differences which exist in sap extracted from identical tissues at various pressures. Among other variations the greater the pressure, the lower was the percentage of solids, although electrolytes in the sap increased. They advise a pressure that causes the pressure gauge on a hydraulic press to barely register. It is stated in their paper that there is no proof that the extract is a true representative of the cell sap, even when expressed and carried at $0^{o}C$. Carrick's work would indicate that this difference may be considerable.

The imbibition theory, in which cold resistance is credited to cell colloids, is not entirely satisfactory in accounting for greater injury through rapid temperature fall; protection from death by cold due to rapid drying of the tissue; or increased tenderness on greater water content. The data submitted in this work show that increased water content is not always accompanied by increased tenderness nor did the imbibitional theory hold good as an explanation of cold resistance. This does not exclude certain factors from standing as measures of cold resistance, yet it does not necessarily follow that such factors are more than indications of the hardened condition. Moreover, as previously mentioned, it remains to be proved that sap extracted is representative of the cell sap or that such samples are suitable for intercomparison. Juice from tissues varying in cold resistance might be reasonably expected to be affected in different degrees and ways by laboratory methods.

Refinement of method, such as will reduce disorganization of and changes in the material used, is desirable before consistent results can be hoped for.

The theory of ice pressure as a cause of death through low temperature apparently explains more of the findings of hardiness and correlated studies than any other. However, it does not necessarily follow that freezing to death always results from the same cause, or that the same factor or group of factors are always responsible. Different methods of inducing resistance to low temperature apparently bring into play different factors which are responsible for this result.

SUMMARY.

1. There was little correlation and no consistent relation between percentage of water in the tissue, freezing point depression of expressed sap, rate of tissue dehydration and the killing temperature with cabbage and tomato plants.

2. The percentage of bound water (by method used) showed no correlation with the rate of tissue dehydration and if anything, negative correlation with the killing temperature.

3. On acid soils the freezing point depression of expressed sap is higher, the "bound water" lower and with one exception, the percentage of total water lower and solids higher than on alkaline soils.

4. It is evident from the results obtained by different investigators that there is need for standardization and perfection of methods of extracting juice from plant tissues, with the aim, if possible, of maintaining more nearly normal conditions.

5. A study of the gross anatomy of the plants used in 1926 showed distinct differences between hardy and tender plants altho these are not necessarily causes of tenderness or hardiness.

PLATE 1.

Cabbage after exposure at $-7^{\circ}C$.

PLATE 2.

Cabbage after exposure at $-5^{\circ}C$. Plant at right $-6^{\circ}C$.

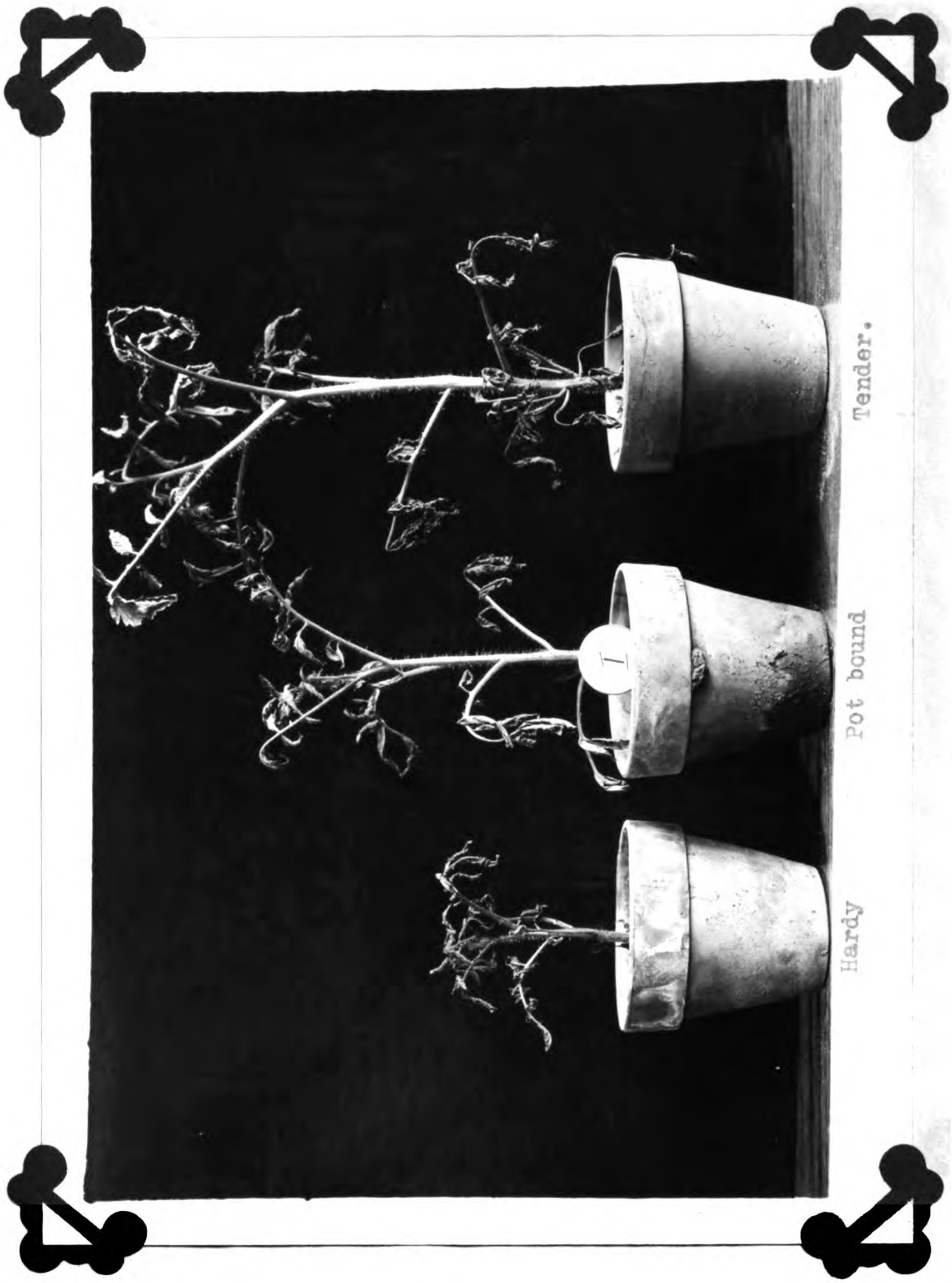


PLATE 3.

Tomato exposed at $-3^{\circ}C$.

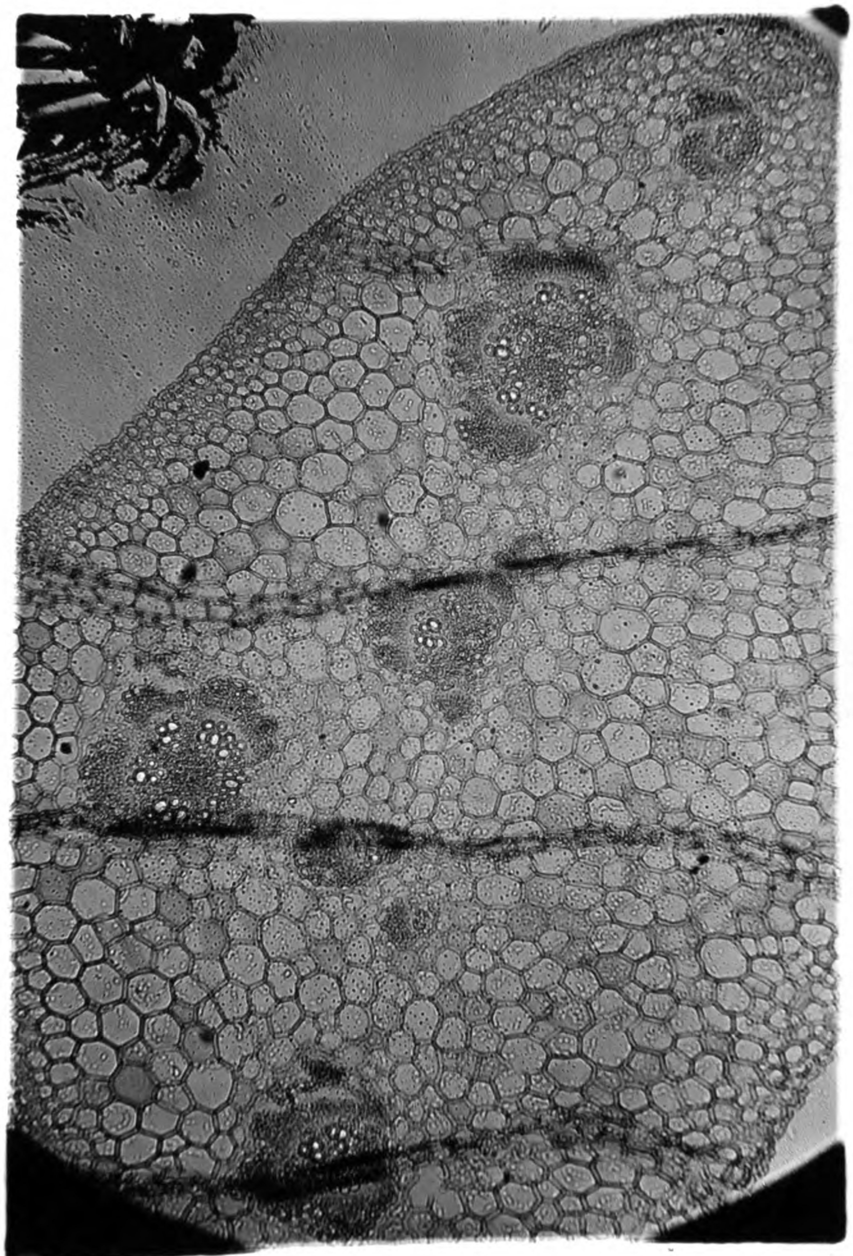


PLATE 4.

Cross-section of Leaf Petiole of Hardy Cabbage.

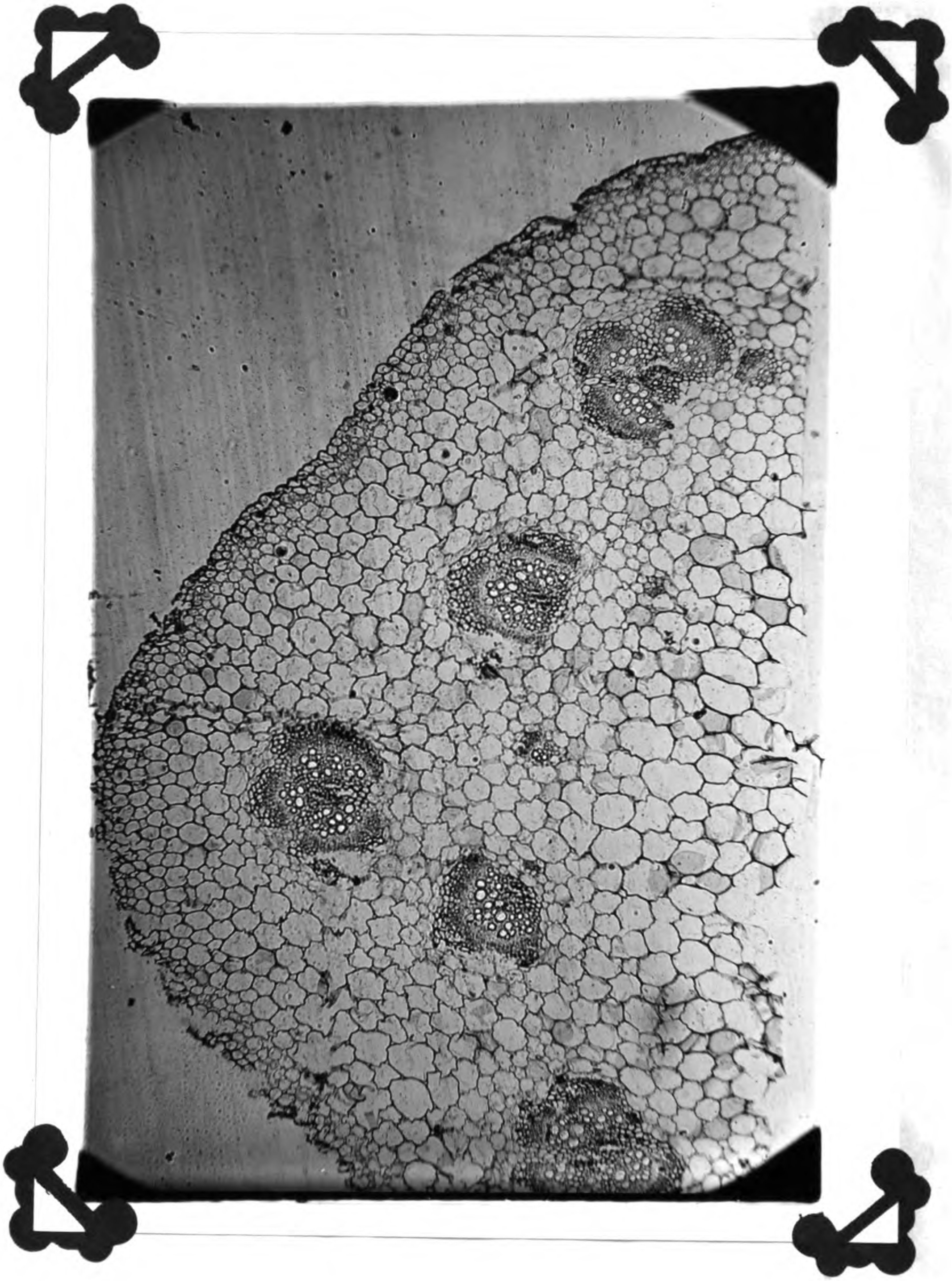


PLATE 5.

Cross-section of Leaf Petiole of Tender Cabbage.

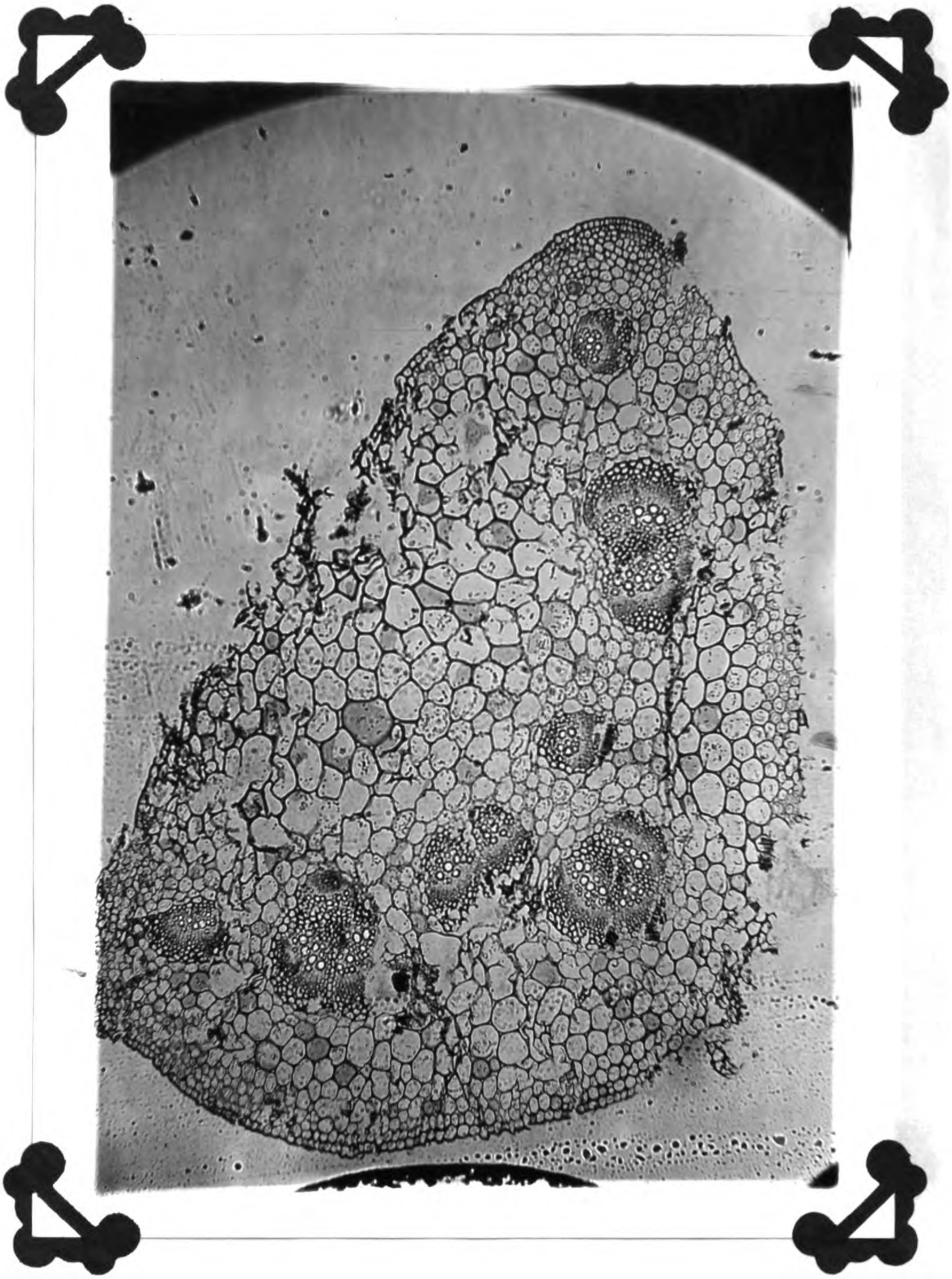


PLATE 6.

Cross-section of Leaf Petiole of Pot Bound Cabbage

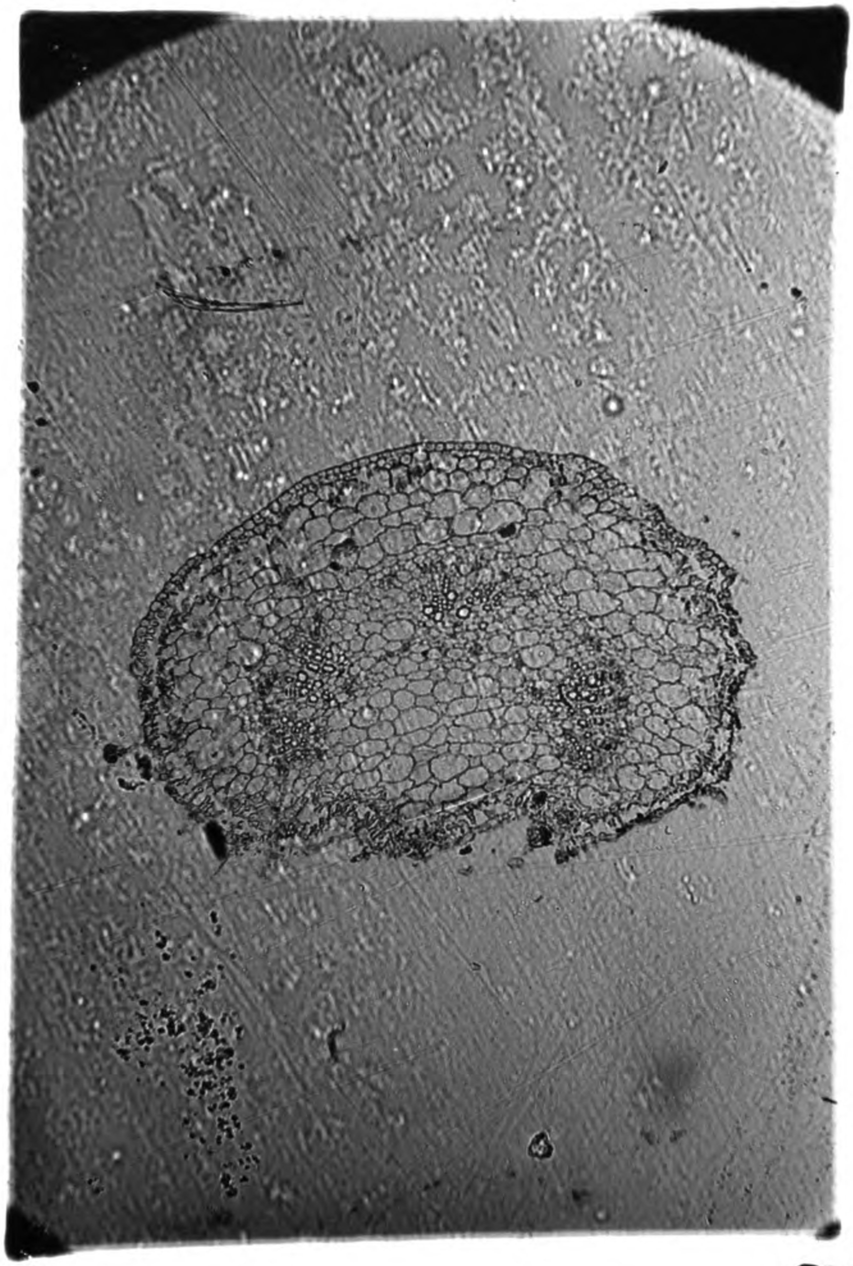


PLATE 7.

Cross-section of Leaf Petiole of Hardy Tomato.

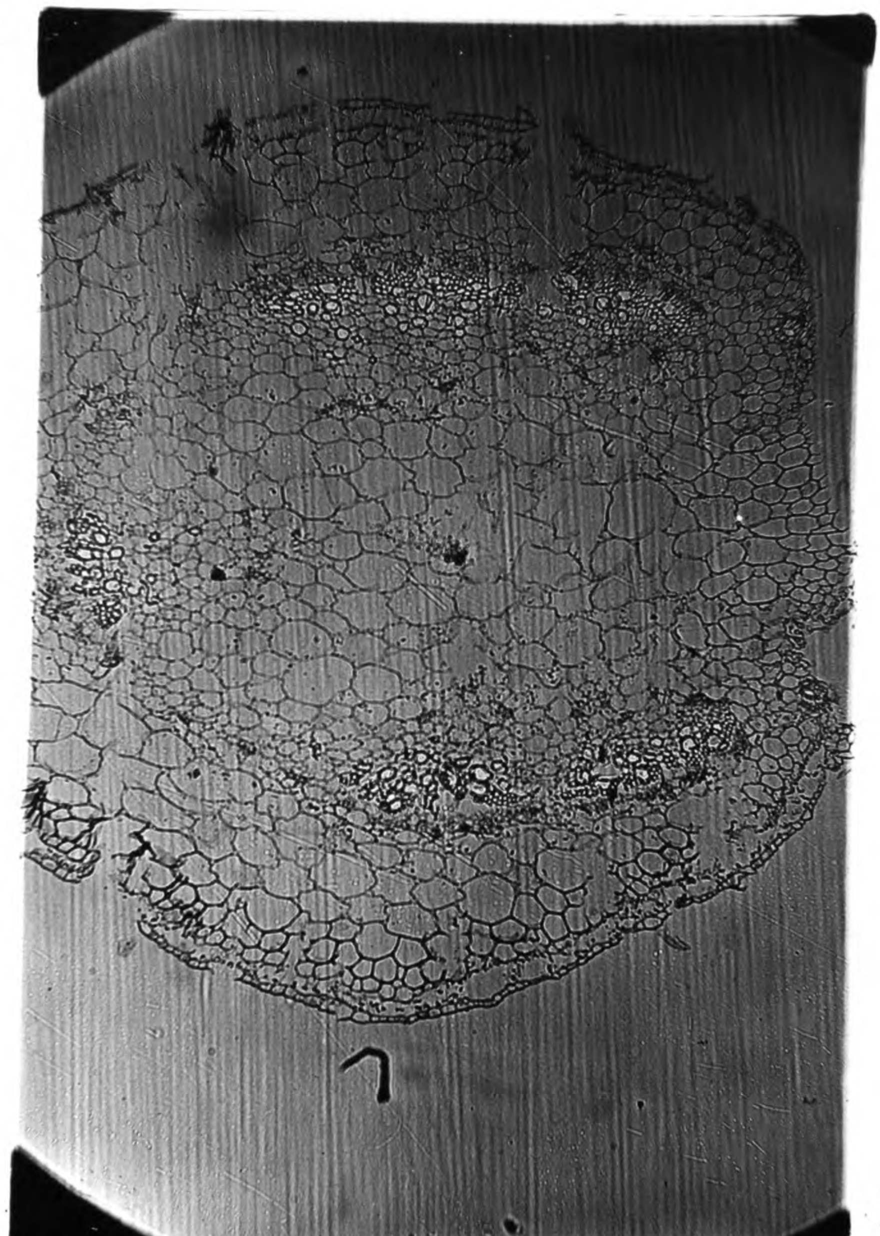


PLATE 8.

Cross-section of Leaf Petiole of Tender Tomato.

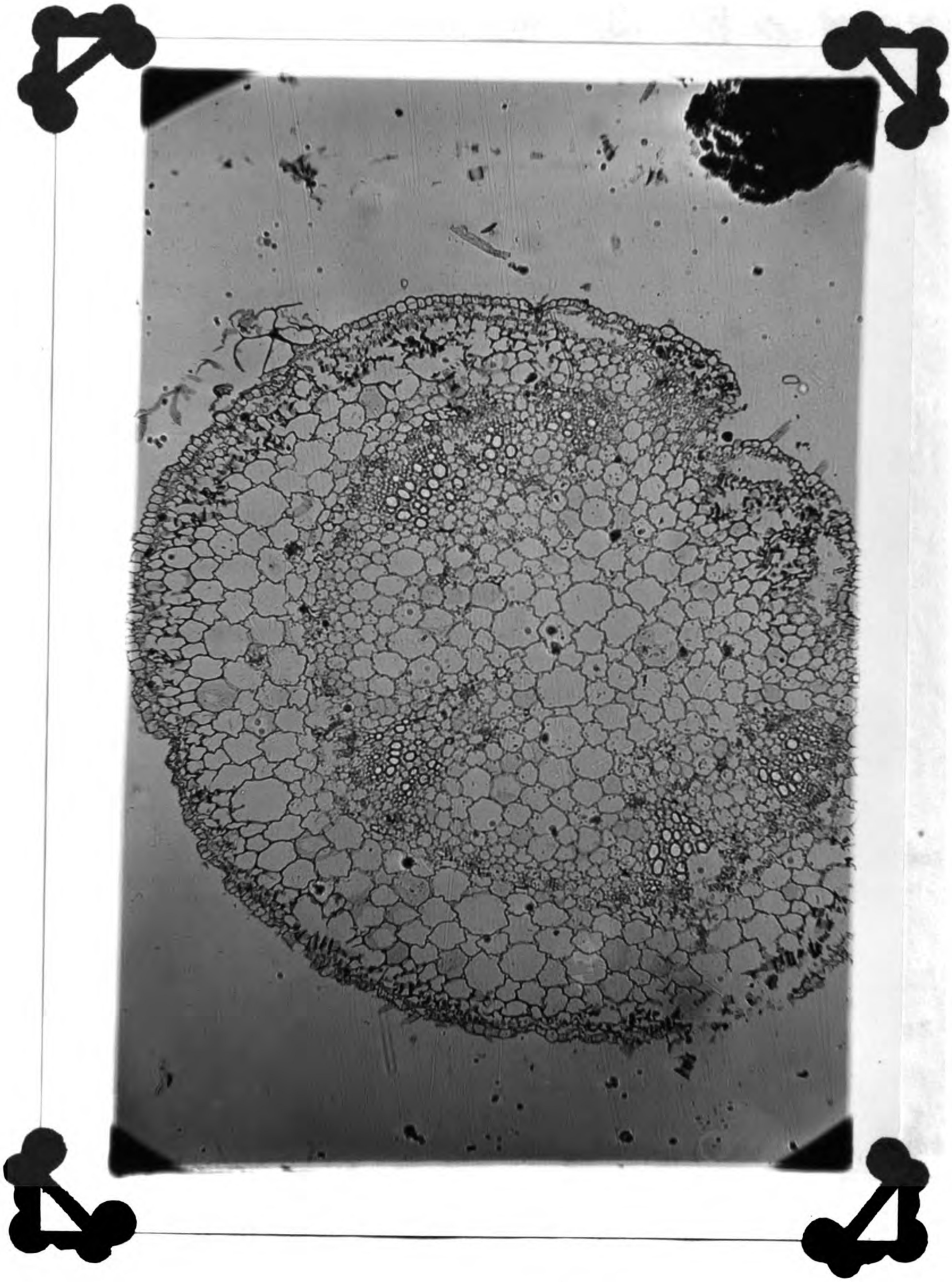


PLATE 9.

Cross-section of Leaf Petiole of Pot Bound Tomato.

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