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Osmotic Factors of Dehardening in *Cornus florida* L.[1]

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ABSTRACT

The killing temperature for cortical cells from the flowering dogwood changes abruptly from −25 C to −15 C during dehardening. Cell sap concentration, minimum critical cell volume, and osmotically inactive cell volume show a progressive change during dehardening, but only cell sap concentration is correlated directly with the killing temperature, showing the same step change. There is a limit to the extent to which hardy dogwood cells can be osmotically reduced in volume. Beyond this limiting volume, the extracellular osmolality can be increased without further volume reduction. Ultimately the cell succumbs, presumably to an osmotic pressure gradient. Nonhardy cells do not exhibit this resistance to shrinkage. The ability to resist volume reduction is probably a crucial factor in the freezing resistance of dogwood cortical cells.

One factor which relates rather precisely to the degree of cell freezing injury is the cell volume reduction which attends freezing. Levitt (5) suggested that the limit of freezing tolerance in plants is a function of the degree of cell contraction. Meryman (9, 10) formulated this concept somewhat differently and has theorized that the onset of freezing injury occurs when osmotic shrinkage has reduced a cell beyond a minimum tolerated volume. Meryman used a number of solutes, both penetrating as well as nonpenetrating, and measured mean corpuscular volume of human red cells after exposure to varying concentrations which simulated freezing exposure. He concluded that the injury to the red cell was unrelated to the absolute concentration of any solute used but was instead related to the removal of a critical proportion of total cell water and the associated reduction in cell volume below a critical minimum. This limit, about 35% of original water content, has been established for invertebrate tissues (13). We have also shown that spinach chloroplast grana can resist freezing injury only when they have become reversibly permeable to concentrated extracellular solute, implicating volume reduction as the source of injury rather than the type or concentration of solute (14).

If volume reduction is the source of injury a cell must find some means to limit osmotic water loss during freezing if it is to acquire freezing resistance. Two ways to achieve this have been reported. The first depends on the presence of "bound" water. If some proportion of cell water does not participate in osmotic events, a greater proportion of osmotically active water must be removed to reach minimum volume, requiring lower temperatures. Second, an increased intracellular solute concentration will extend freezing tolerance, since this will lower the freezing temperature.

In this paper we test the minimum volume hypothesis in a hardy plant. The species chosen was the local flowering dogwood (*Cornus florida* L.) selected because its parenchymal cells have a cellular morphology which allows simple osmometric volume determinations. Although it is related to the northern red-osier dogwood (*Cornus stolonifera* Michx.) which has been shown to be fully tolerant to freezing temperature below −125 C (8), it is considerably less hardy than its northern relative, being killed at −26 C (1). The osmotic factors investigated were the normal cell sap osmolality determined by incipient plasmolysis, the nonosmotic volume of the cell, and the critical minimum volume of the cells. These parameters were measured regularly during the dehardening period and were correlated with killing temperature determinations.

MATERIALS AND METHODS

Since the cortex has been reported as the hardiest of all plant tissues (7), both osmotic and killing temperature determinations were made using this tissue. Because of a report from Weiser's laboratory (12) that the killing temperature of hardy *Cornus stolonifera* varied with the weather, all experimental material was taken from a single dogwood tree to reduce variations which might result from differences in age, physical condition, and microclimate. In fact we found no difference in other (unpublished) studies on dogwoods from a number of clones in the Bethesda area. We used only twig sections formed during the growing period of the previous year.

Since freezing can be simulated by placing cells in hypertonic solutions of nonpenetrating solutes, we chose this means to study cell osmotic responses. Because calcium normally does not penetrate the cell membrane, we used a series of $CaCl_2$ solutions ranging in concentration from 0.13 M to 2.12 M,[3] with freezing points from −0.66 C to below −20 C. Using a modification of the procedure of Levitt and Scarth (7), cortical parenchymal tissue was stained in 0.008% neutral red dye in water to improve visibility under the microscope. A stock solution of 0.04% neutral red was stored at 4 C and freshly diluted for each experimental run.

The epidermis from small twig sections was removed with a scalpel and longitudinal strips of the underlying parenchymal tissues were immediately placed in a well slide containing the neutral red solution. The segments were completely stained after 30 min. Sections of the stained tissue were teased out under a 30X stereomicroscope with dissecting needles and transferred for microscopic observation to a perfusion chamber constructed from a hemocytometer. A hypodermic needle was cemented in the gutter of the hemocytometer and attached to a connecting tube leading to a waste reservoir maintained under slight suction. Each solution of $CaCl_2$ was introduced at the front edge of the hemocytometer coverslip and was drawn by capillarity between the coverslip and the body of the hemocytometer. The

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[3] Concentrations are given as osmolal, which are ideal molal concentrations based on freezing point depression. At room temperature, a solution of 1 osmolal concentration has an osmotic pressure of about −24 atm.

excess flowed into the gutter of the hemocytometer and was removed by the suction apparatus. Each solution of progressively higher osmolality was introduced three times at 1-min intervals to approach equilibrium. When a $CaCl_2$ concentration was reached which produced some plasmolysis, particular care was taken to locate a cell whose plasmolysis conformed as nearly as possible to the stipulations set down by Höfler (3). These stipulations are that the cell be cylindrical with a circular (or elliptical) cross-section and that as the protoplast pulls away from the cell wall its ends be hemispherical. Suitable cells were located and were photographed through a photomicroscope onto 35 mm film. If, during the process of plasmolysis the protoplast shape deviated drastically from a regular geometry, another cell was chosen and the procedure repeated. The dimensions of the cells were determined to ±0.25 mm from enlargements with a total magnification of about 1000 diameters. A typical plasmolyzed cell is seen in Figure 1. Our observations showed that the ends of the cells regularly departed from hemispheres so that the calculations produced an estimate of cell volume which was too small. Since the cell diameter remained virtually constant throughout each plasmolytic series, we treated the plasmolyzed protoplasts as right cylinders. While this produces cell volume estimates which are several percent too large relative to Höfler's equation, the pattern of osmotic behavior is not distorted.

The osmotic behavior of each cell was plotted using the Boyle-van't Hoff relationship:

$$V = nRT/\pi + b$$

where n = osmolal concentration of osmotically active intracellular solute; π = the osmolal concentration of the external solution; R = the gas constant; T = the absolute temperature; b = volume of the osmotically inactive portion of the cell.

If nRT and b remain constant, cell volume will vary as a linear function of $1/\pi$. The best fit straight line for the points in each Boyle-van't Hoff plot was determined according to the principle of least squares. By substituting into the linear regression equation, the concentration at incipient plasmolysis could be estimated as the π for each curve at $V = 100\%$. The volume, b, which contains both cell solids and osmotically inactive water, cannot be directly measured. Rather, it is defined as lim. V.

$$\pi \to \infty$$

To determine the killing temperature, a twig of 0.3 to 0.5 cm diameter was cut into several sections 1.5 to 2 cm long. Each section was placed in a test tube and immersed in a refrigerated alcohol bath at preset integral degree temperatures (±0.05 C) for 75 min. Thermocouples inserted into twigs showed that temperature equilibrium to the bath was approached after about 12 min, so that exposure to the low temperature was 1 hr. The twig was removed and left at room temperature in a small Petri dish for approximately 20 min. Small longitudinal strips of cortical parenchyma tissue were removed and stained with neutral red dye as previously described and perfused with a concentrated 2 M $CaCl_2$ solution. The ratio of plasmolyzed or live cells to unplasmolyzed or killed cells was counted in several microscopic fields for each sample. The killing temperature was defined as the temperature at which 95% of the cells observed did not plasmolyze when exposed to a 2 M $CaCl_2$ solution.

RESULTS

From the initiation of this study on March 20, 1971, until April 15, 1971, the killing point of the cortical tissue remained constant at −25 C. The killing point was sharply defined; about 10 to 15% of the cells plasmolyzed when frozen to −24 C, while after exposure to −26 C, none of the cells plasmolyzed in the

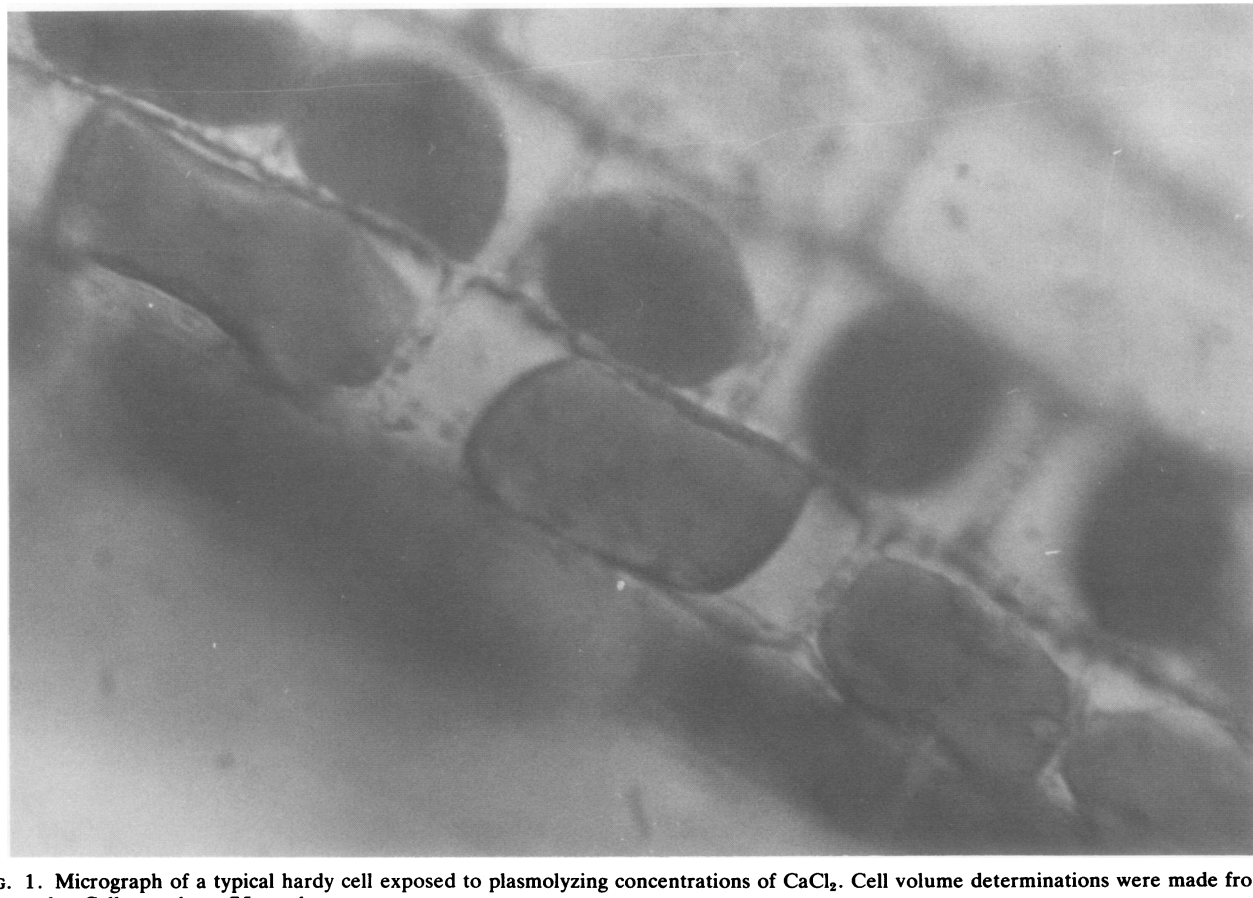

FIG. 1. Micrograph of a typical hardy cell exposed to plasmolyzing concentrations of $CaCl_2$. Cell volume determinations were made from such photographs. Cell was about 75 μm long.

concentrated solution of $CaCl_2$. During the week of April 23, there was an abrupt rise in the killing temperature to −15 C. Here also at temperatures 1° higher or lower than −15 C the cells responded in a manner similar to the hardy cells at −25 C. The killing temperature from April 23 until the end of this study on June 30 remained constant at −15 C. The dry weight of hardy tissue was 50% of its fresh weight and it did not change with dehardening.

The manner in which cells responded osmotically to $CaCl_2$ solutions and the changes that occurred during dehardening may be seen in Figure 2. In each case, the same general form of curve is seen. As the extracellular osmotic potential increases from right to left the cells begin to plasmolyze and cell volumes decrease in good agreement with the Boyle-van't Hoff prediction until a departure from linearity is seen. At this point minimum volume is attained. While further increases in concentration were tolerated by the cells, no further volume reduction was observed as is seen by the plateau portion of the curves. In each case, the concentration at incipient plasmolysis, osmotically inactive volume (b), and minimum volume were determined and are given in Table I.

During the dehardening process, the concentration at incipient plasmolysis decreased steadily from a high of 961 milliosmols to 459 milliosmols as seen by the right shift of the curves in Figure 2. A sharper decrease occurred during the week hardiness broke than before or after. If the values during the hardy

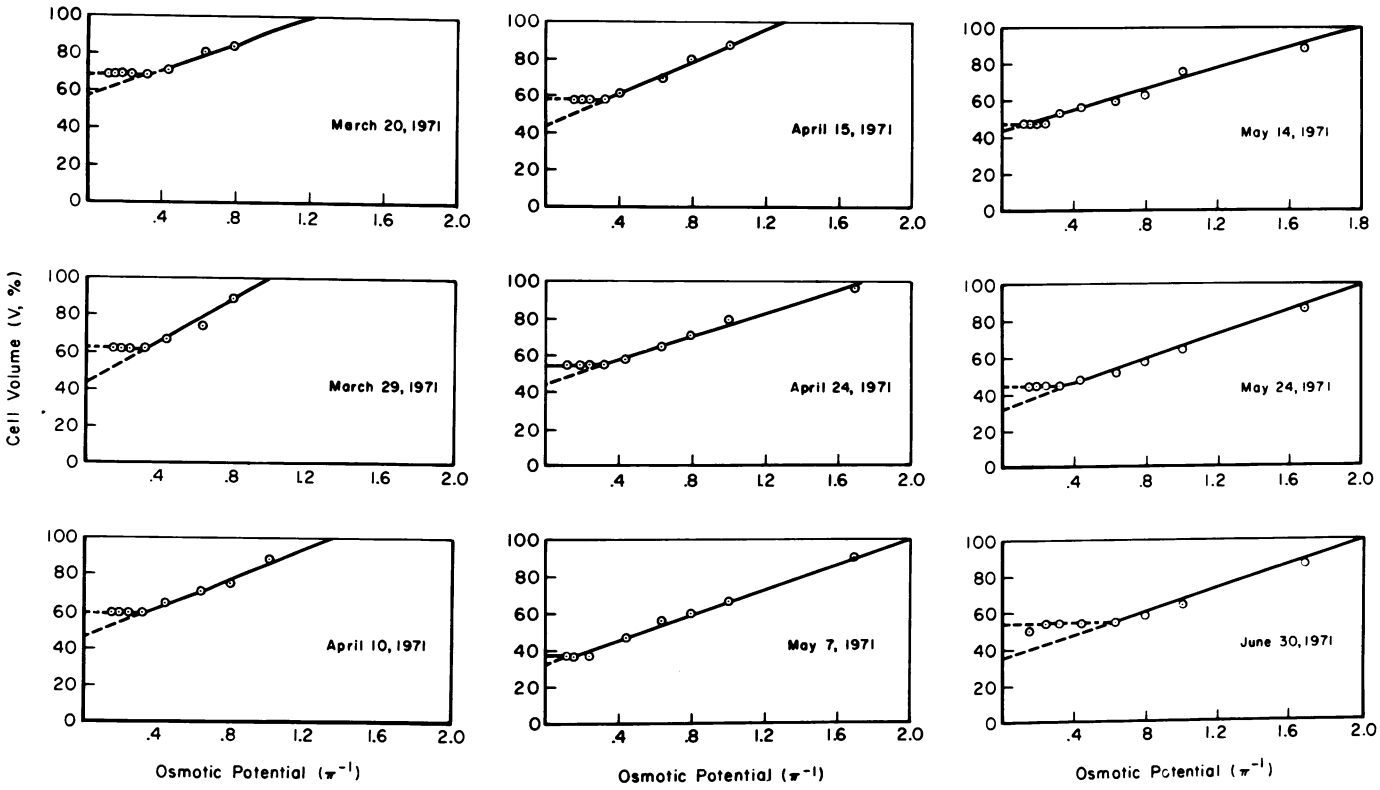

FIG. 2. Boyle-van't Hoff plots of cell plasmolysis during exposure to $CaCl_2$ solutions at room temperature. Cell volume is relative to volume of turgid cell, and osmotic potential is that of plasmolyzing solution, as osmolal^{-1}. During dehardening, which occurred the week of April 24, the point of incipient plasmolysis decreased sharply, and the resistance to shrinkage that gave the volume plateau at high concentrations almost disappeared.

Table I. *Osmotic Determinations*

Date (1971)	Osmotically inactive Volume b, %	Point of incipitent plasmolysis	Minimum volume,%	Killing Temperature
20 March	57.3	814 mOsm	69	
22 March				-25 C
29 March	43.2	961	62	-25
8 April				-25
10 April	46.6	738	59	
15 April	43.8	769	58	-25
23 April				-15
24 April	45.9	574	54	
30 April				-15
7 May	33.4	502	37	-15
14 May	43.9	487	47	
21 May				-15
24 May	33.5	472	45	
30 June	35.7	459	52	-15
	$p<.02$	$p<.001$	$p<.001$	

Dashed line indicates time at which hardiness was broken.

and nonhardy period are statistically compared, the difference is highly significant ($P \ll 0.001$). Similarly, b showed a general decreasing trend, from 57.3 to 35.7%, during dehardening. The break in this trend during the week of dehardening is unexceptional. Nonetheless, the difference between hardy and nonhardy values was significant ($P < 0.02$). Finally, the minimum volume also decreased during dehardening from a high of 69% to a low of 37%. The difference between hardy and nonhardy groups was highly significant ($P < 0.001$). The plateau generally seemed to be shorter in unhardy cells than it had been in hardy cells.

During the hardy period, most cells in the microscopic field could be exposed to the highest concentration of $CaCl_2$ (2.1 M; freezing point below −18 C) and deplasmolyzed stepwise back to full turgidity. During deplasmolysis the cells followed the same curve back to full turgidity as that down to the plateau, indicating that the plateau had not been caused by solute entry into the cells. In all cases when the cells reached full turgidity they could again be plasmolyzed which we interpreted as meaning that the cells had survived the exposure. Nonhardy cells could not be plasmolyzed past 1.6 M $CaCl_2$ (freezing point −12.3 C) with subsequent return to full turgidity on deplasmolysis. When nonhardy cells were exposed to concentrations of $CaCl_2$ greater than 1.6 M, the protoplasts exploded during deplasmolysis and released dye before they reached full turgidity. In these cases the cells were irreversibly damaged. In both hardy and nonhardy cells, the tolerated plasmolysis in $CaCl_2$ solutions was somewhat less than that predicted from the freezing experiments.

The Boyle-van't Hoff plots of hardy cells show that contraction of the protoplasts abruptly ceased at concentrations much more dilute than those to which the cells could be safely exposed with return to full turgidity on deplasmolysis.

DISCUSSION

The flowering dogwood does not survive in regions where the temperature normally goes below −26 C (1). This natural killing temperature is quite consistent with the experimental killing temperature in this study. The killing temperature of nonhardy trees would not be as low as the −15 C we found since the most sensitive part of the tree during the nonhardy season would be the leaves not the cortical tissue.

If the major cause of freezing injury is osmotic, the sudden change in resistance at dehardening should be accompanied by an equivalent change in osmotic parameters. The abrupt change in cell sap concentration correlates well with the sharp change in hardiness of the flowering dogwood. The osmotically inactive volume and the minimum volume do not change as dramatically as the break of hardiness and while they are obviously important to hardiness, it is hard to implicate them as a major cause of the loss of hardiness. Observation of the entire tree during the loss of hardiness did not show any obvious morphological changes. Flower and leaf buds still appeared quiescent, and bud break did not occur for 2 weeks. The fact remains that a 10° rise in killing temperature occurred during the period of April 15 to April 23 and that, except for cell sap concentration, no other obvious corresponding change was seen. Li *et al.* (8) found a killing point differential of 50 F over a similar period of time in the red-osier dogwood. The osmotic parameters which we studied are incomplete since cell sap concentration would of itself be inadequate to explain the change.

Bound water has been frequently invoked to explain freezing resistance in living cells. Our data support this. In the hardy cells approximately 48% of the total volume of the cell is not osmotically active. During this study we performed one plasmolysis test on hardy red-osier dogwood from the University of Wisconsin arboretum which has a substantially lower killing temperature than the flowering dogwood. We found a b value of about 70%. This is consistent with the observation that osmotically inactive volume makes a considerable contribution to hardiness. Unfortunately osmotically inactive volume cannot be resolved into the proportion of the cell volume which is bound water and the proportion which is cell solids, and it can be argued that the high b values during the hardy period are a result of increased amounts of cell solids rather than bound water (4, 11). We have accumulated considerable data on this issue, but have not resolved it.

The most interesting observation in this study was the behavior of the cells when they reached their minimum volume. Hardy cells could be exposed to still higher concentrations without injury and without a volume reduction. This plateau shortened abruptly in nonhardy cells and is a major factor in the freezing tolerance of this species. Williams and Meryman (14) found, when exposing spinach grana to freezing or hypertonic stress in the presence of cryoprotective concentrations of sorbitol, that exceeding minimum volume was prevented by an influx of extracellular solute. Our experiments showed no such leakage of solute into the cells since the volume curves were retraced as extracellular osmolality was reduced. If increasing extracellular concentration does not result in decreased volume and if there is no evidence of solute influx, we are left with an anomaly. Barring some extraordinary intracellular changes, there can then no longer be osmotic equilibrium across the membrane in the plateau portion of the curves. There must then either be a complete impermeability to water or there must be a mechanical force present within dogwood cells which resists external osmotic pressure and prevents cell water loss (15). We favor the second possibility.

Since the total pressures experienced by a cell membrane must be equal, a resistance to volume reduction on one side will require a reduced osmotic pressure from that side. Accordingly, Baker (2) showed that *Nitella* cells, when exposed to increasing concentrations of nonpenetrating salt, develop subatmospheric intracellular pressures as the cells begin to plasmolyze. Apparently *Nitella* cells resist the volume reduction associated with the loss of intracellular water. We are proposing that hardy dogwood cells also resist volume reduction, particularly after the cells have plasmolyzed to a critical volume. The nature of this resisting factor is unknown but we propose that its function is to provide a means of obviating dehydration injury. As concentrations outside the cells increase, resistance to shrinkage maintains cell volume but at the cost of an increasing concentration difference between inside and outside. The difference eventually becomes greater than the cells can tolerate, membrane integrity is lost and the cells are damaged. We suggest that this mechanism be added to water "binding" and augmentation of cell solute, the mechanisms long acknowledged to extend freezing tolerance.

We have no precise way to calculate how each of the osmotic influences might affect the degree of hardiness if they could be sorted out. Increased cell sap concentration is essential to cryoprotection although Levitt (6) has shown that it is ineffective by itself in reducing killing temperatures more than a few degrees. The phenomena of water "binding" and plateau formation seem to bear the burden of protecting dogwood against freezing injury.

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