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Freezing Tolerance of Onion Bulbs and Significance of Freeze-Induced Tissue Infiltration[1]

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Because of their high water content (80–90%), onion bulbs are usually regarded as freezing-sensitive organs. In the fall of 1974, onions grown in the field at the University of Minnesota Sand Plain Experimental Field at Elk River froze when the air temperature dropped to −10°C for a few hours following 5–6 nights with minimum temperatures near 0°C. To our surprise, however, more than 95% of the frozen bulbs remained healthy and normal after they were transported to a storage area and thawed.

Onion bulbs therefore possess a much higher degree of freezing tolerance than previously realized and their exact tolerance needs to be determined. Fortunately, they are a standard material for cell physiological investigations and have also long been used both in our laboratories and elsewhere. There are many reasons for this choice (9). The cells are quite large, and the inner epidermis of the scales can be peeled off easily for microscopic observations as a cell monolayer. The variability between the bulbs of a variety is small. They can also be kept in cold storage for several months without major changes in cell properties (6).

Onion bulbs should therefore offer highly useful material for investigating the physiology of freezing injury and tolerance. So far, only the outer and inner epidermis of the onion scale seem to have been used for testing freezing rate and survival (7, 8), and no detailed physiological investigation has been attempted.

MATERIALS AND METHODS

We used *Allium cepa L.*, cv. "Downing Yellow Globe" grown on coarse sandy loam soil at the University Sand Plain Experimental Field.

Inner (or Adaxial) Epidermis

The upper and lower quarters of the onion bulbs were discarded and the middle portion was used. The third healthy scale (counting inward from the outermost fleshy scale) was selected, and the inner epidermis was peeled off with a pair of forceps, carefully avoiding bending. Five 1-cm² pieces of epidermal layer were prepared and transferred to a series of test tubes lined with wet filter paper. The epidermal pieces were placed on the filter paper. These test tubes were then transferred to a cooling bath containing an antifreeze solution consisting of an ethylene glycol base.[4]

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[4] Freezite–antifreeze and summer coolant, W. H. Barber Oil Co., Minneapolis, Minnesota.

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The temperature was lowered at the rate of 1°C/hr. Inoculation occurred spontaneously because of the wet filter paper. When the desired temperature was reached, the epidermal strips were thawed slowly by removing the test tube and burying it in ice contained in a styrofoam box. After about 12 hr, microscopic observations of the epidermis determined cell survival. Protoplasmic streaming and occurrence of plasmolysis in hypertonic solutions of mannitol were used as criteria for cell survival. Temperatures as low at −21°C were used.

Whole Onion Bulbs

Medium-sized bulbs (diameter between 65 and 75 mm) weighing about 130 g were selected and held in a cardboard box which was then placed inside a freezer in which the temperature could be regulated. This arrangement helped to eliminate the temperature fluctuations which often occur in freezers. A continuous record of the temperature inside the onion was obtained by inserting into the middle of the onion a thin wire copper–constantan thermocouple connected to a recorder.

Two types of freezing regimes were used: Regime I: Bulbs were first frozen to equilibrium at −4°C (time needed, 116 hr), and then the temperature of the freezer was lowered 5°C every 4 hr. Temperatures as low as −25°C were studied. Regime II: Bulbs were transferred directly from storage to freezers maintained at −4 and −11 ± 0.5°C and were kept frozen up to 12 days.

Supercooled onions were induced to start freezing by shaking or by touching the bulbs with a cold pair of forceps (temperature −40°C) at the point of insertion of the thermocouple.

For observation of injury, frozen onions were removed, after freezing to a selected temperature for a desired length of time, and transferred to a styrofoam box for slow thawing. After thawing, onions were cut in half longitudinally and the infiltration (water-soaked appearance) of the individual scales was estimated visually. Epidermal and parenchyma cells from infiltrated scales were observed for protoplasmic streaming and plasmolysis. The cells which showed protoplasmic streaming and/or plasmolysis were counted as living cells.

RESULTS

1. Inner Epidermis

Although there was some variability among different bulbs, about 60 to 80% of the epidermal cells, frozen at a cooling rate of 1°C/hr survived to −20°C. After freezing at −21 or −22°C most cells appeared dead and above −18°C all cells

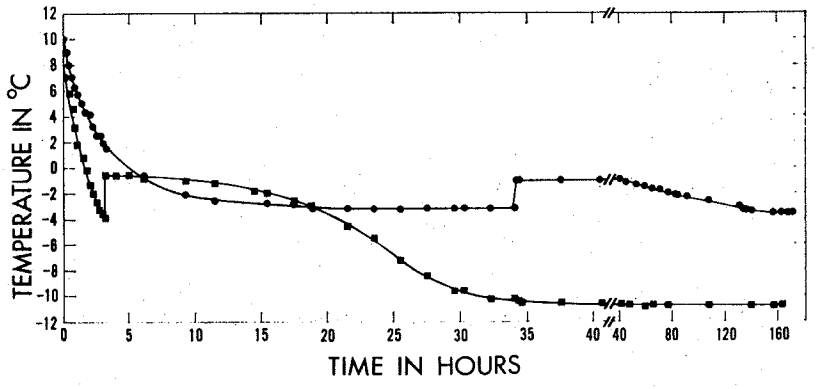

FIG. 1. Freezing curves. Temperatures in onion bulbs when transferred directly to air temperatures of −4 ± 0.5°C (●——●) and −11 ± 0.5°C (■——■).

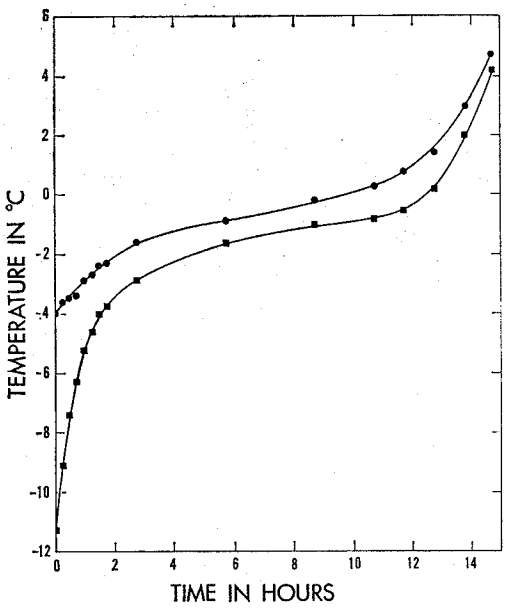

FIG. 2. Thawing curves. Temperature in onion bulbs when transferred from -4 ± 0.5°C (●——●) and -11 ± 0.5°C (■——■) to a styrofoam ice box for thawing.

were living. More rapid cooling rates (up to 4°C/hr gave the same results. These experiments were repeated at least five times.

2. *Whole Bulbs*

(*a*) *Freezing.* Typical freezing curves for whole onion bulbs (freezing Regime II) are shown in Fig. 1. Bulbs exposed to −4°C cooled down to about −3°C in approximately 19 hr. If undisturbed, these bulbs remained supercooled at this temperature for at least 20 hr at which time they were subjected to freezing. As freezing began, the bulbs warmed up to about −1°C and remained there for about 7 hr. After this time, the bulbs cooled slowly and finally reached −3.8°C about 120 hr after nucleation.

Bulbs exposed directly to −11°C cooled to −4° in 3 hr, then nucleated spontaneously (Fig. 1). As freezing continued, the bulbs warmed to about −1°C and remained at this temperature for about 7 hr. After this period the bulbs cooled to −11.4°C in about 35 hr after nucleation. This experiment was repeated 10 times with similar results.

Under freezing Regime I, only about 10% of the bulbs nucleated spontaneously, while the rest remained supercooled and had to be nucleated artificially to initiate freezing. It was found that with the applied cooling rate (5°C/hr) the bulbs themselves cooled down 1°C/hr.

(*b*) *Thawing.* In all cases the frozen bulbs were taken out of the freezer and transferred to an ice box for thawing. Typical thawing curves for bulbs frozen to −4 and −11°C are shown in Fig. 2. The bulbs warmed up very rapidly during the first 2 hr to about −2°C in the case of the −4°C treatment and to about −3.5°C in the case of the −11°C treatment. Following this rapid rise, both curves rose more gradually and ran almost parallel, for about 10 hr, to −0.5°C, indicating that bulbs frozen at these two different temperatures thawed in a very similar manner 2 hr after the initiation of thawing. This thawing pattern was found repeatedly in 10 different bulbs.

(*c*) *Cell survival.* In freezing Regime I, at temperatures of −4, −9, −15, −20, and −25°C two bulbs were removed from the

TABLE 1

Effect of Short-Duration Freezing Stress on Extent of Infiltration and Cell Survival of Onion Bulb Scales after Thawing[a]

Freezing temperature (°C)	Extent of infiltration of onion scales (%)		Percentage of cells living in scale tissue	
	Days after thawing		Days after thawing	
	0	3	0	3
−4	30[b]	None	100	100
−9	60	None	100	100
−15	80	15	100	100
−20	100	100	95	90
−25	100	100	5	0

[a] After freezing at −4°C (time needed, 116 hr), the temperature was lowered 5°C every 4 hr.

[b] All figures are within ±5%.

freezer and thawed over ice. The degree of infiltration increased from 30 to 100% as the freezing temperature was lowered from −4° to −20°C (Table 1). Almost all the cells in the tissue, even at 100% infiltration, were living (Table 1). There was therefore no relationship between the extent of infiltration and the relative number of dead cells in the thawed tissue. These results agree with the recovery observed after injury by salt stress, which leads to exosmosis of ions (5).

After observation for cell survival, the thawed and halved bulbs were left at 5 and 25°C to see if injury increased or decreased with time. It was found that in bulbs frozen to −4 and −9°C infiltration disappeared completely in 3 days (Table 1). In the case of bulbs frozen to −15°C, infiltration disappeared from all except the first two scales which remained infiltrated. All the scales of bulbs frozen to −20 and −25°C remained completely infiltrated. This shows that the injury which led to infiltration of the tissue could be reversed spontaneously in bulbs frozen to −4, −9, and to a large extent in those frozen at −15°C. However, the injury was not reversed in the cases of −20 and −25°C. Thawed bulbs kept at +5°C recovered much better than those kept at +25°C. Further, in most of the cases many cells survived −20°C. In bulbs frozen to −25°C almost all the cells were dead.

In freezing Regime II, at both temperatures (−4 and −11°C), the extent of infiltration of onion bulb scales observed immediately after thawing increased when the freezing period was extended from 6 to 12 days (Table 2). In general, infiltration was much greater in bulbs frozen at −11 than at −4°C. The thawed and halved bulbs were left at +5°C and observed again after 3 days. It was found that in bulbs frozen at −4°C, infiltration disappeared completely, whereas it increased in bulbs frozen at −11°C. This indicates that the cell injury in bulbs frozen at −4°C was reversed, but in bulbs frozen at −11°C this injury increased with time after thawing. All cells in each treatment were living (i.e., could be plasmolyzed) at both 0 and 3 days after thawing. This confirms the results in Table 1, showing that infiltration of tissues is not a measure of the number of dead cells. Cell damage that leads to irreversible infiltration in bulbs frozen at −11°C did not result in cell death even 3 days after thawing.

TABLE 2

Effect of Prolonged Freezing Stress on Extent of Infiltration of Onion Scales after Thawing

Freezing temperature (°C)	Number of days onions kept frozen	Extent of infiltration of onion scales (%)	
		Observation time after thawing (days)[a]	
		0	3
−4	6	30[b]	None
	12	60	None
−11	6	75	100
	12	90	100

[a] All the cells from each treatment were living at both observation times.

[b] All the figures are within ±5%.

3. *Repeated Freezing and Thawing*

The effect of three successive freezings and thawings was studied. Bulbs were frozen by exposing them directly to −4°C (Fig. 1). When equilibrium was reached (116 hr after nucleation) they were thawed over ice for 12 hr. This was repeated three times using the same bulbs. At each cycle two bulbs were tested. No effect of repeated freezing and thawing was found on extent of infiltration. Bulbs frozen one, two, and three times all showed about 30% tissue infiltration. This infiltration disappeared in all the three cases in 3 days after thawing.

DISCUSSION

Death by any method, in the absence of tissue dehydration, results in a water-soaked appearance of leaves. This is due

to infiltration of the normally air-filled intercellular spaces with an aqueous liquid originating from cell sap and protoplasm. Investigators of freezing injury frequently assume that the converse is also true: If the leaves of a thawed plant are infiltrated, their cells are dead. Theoretically, however, this conclusion is not necessarily valid. In the case of most stresses, infiltration occurs because the cells are killed by the stress and lose their property of semipermeability. The cell sap content therefore leaks into the intercellular spaces. In the case of freezing, however, there is another factor. Since ice forms extracellularly under normal (slow) rates of freezing, this infiltration may be at least partly due to the thaw water remaining in the intercellular spaces for some time after thawing is complete. Since ice consists of essentially pure water in the solid state, the thaw water should be immediately reabsorbed osmotically by the cells if they are completely uninjured. Consequently, such a completely uninjured tissue should not appear watersoaked (infiltrated) on thawing. If infiltration is observed, it must be a resultant of both factors, *inter*cellular ice formation and injury to the cells. The question is: How much injury? Many investigators assume that the percentage of a tissue that is observably infiltrated on thawing, is a measure of the percent of cells killed (1, 3, 10). Others (4) assume that the leaves must be left at 0°C for several days before attempting such an estimate. In no case have these investigators tested this assumption directly by microscopic examination of the cells for viability.

It is interesting to note that Germ (2) as early as 1938 had already observed the disappearance of intercellular liquid (infiltration) on careful thawing after freezing onions to −2°C. However, he reported that onion cells die at −5°C.

In this investigation, direct tests of cell viability were used, and it was clearly shown that all the cells of the infiltrated tissues were alive on thawing, as long as the bulbs were allowed to attain equilibrium at freezing temperatures no lower than −18°C. Infiltration of thawed tissues, therefore, must not be equated with cell death. This may be one reason why the freezing tolerance of onion bulbs has been previously underestimated. The observation of tissue infiltration immediately after thawing was probably accepted as evidence of cell death, and the bulbs were discarded without waiting for recovery.

There is another important conclusion from these observations. One component of the freezing tolerance of onion bulbs is a healing process that occurs after thawing and that results in the disappearance of freeze-induced tissue infiltration.

SUMMARY

(1) In spite of the high water content of the onion bulb, most of its cells are alive on thawing after slow freezing to −20°C, and all are alive after slow freezing for several hours to any temperature above −18°C. They even survive 12 days at −11°C.

(2) All the tissues of onion bulbs are infiltrated on thawing, after slow freezing to temperatures of −20°C, and almost a third of the tissues are infiltrated even after slow freezing to only −4°C.

(3) Infiltration of thawed tissues, therefore, is not a criterion of cell death.

(4) As further evidence of this conclusion, bulbs frozen at −4°C and showing 30–60% infiltration completely recovered after 3 days at 5°C and no longer showed any infiltration.

(5) In opposition to this recovery from infiltration, onion bulbs frozen for many days at −11°C and showing 75% infiltration on thawing are 100% infiltrated after 3 days at 5°C.

(6) One component of the freezing tolerance of onion bulbs is, therefore, a healing process that occurs after thawing. Con-

versely, if frozen to a temperature below its full tolerance, the injury progresses after thawing is complete.

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