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Protective modification of freeze stress in plant tissue [1]

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Abstract

Arabinoxylan mucilages from cell walls of hardy winter cereal plants inhibit the initial growth of ice crystals. These polymers affect the kinetics of freezing by adhesion to ice through interfacial liquid. The energy of polymer hydration decreases the latent heat of freezing by reducing the activation energy of melting. The decrease is a measure of adhesion energy. Other hydrated substances including the plasmalemma also equilibrate with freezing by adhesive interactions. Adhesion can produce lethal stress by −10°C. The adhesive stress can be reduced to osmotic stress by release of solutes from the protoplasts into the intercellular space. The interfacial tension in winter cereal plants is reduced by hydrolysis of fructan to sugars.

INTRODUCTION

Freeze preservation of foods may involve some of the problems that affect survival of plants in winter. Freezing of water in plant tissue causes a sequence of physical stresses to develop as the temperature decreases. These can be analyzed in a variety of ways. The following description of imaginary resonance between different forms of energy was used to distinguish stress components in winter cereal plants [1].

If the Maxwell–Boltzmann frequency distribution is assumed to represent the distribution of kinetic energy that can be exchanged between molecules in a collision (the instantaneous temperature of individual molecules), then the latent heat is the translational kinetic energy that a water molecule in ice at the liquid interface must convert to potential energy to become a molecule of liquid [2]. The difference in potential energy between ice and liquid is due to the difference in bond energy and degrees of freedom, especially for rotational kinetic energy. Thus only ice molecules with translational energy greater than the activation energy of

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melting can escape, and the energy of melting is then the integral of the product of the excess energy and the frequency. Conversely, only liquid molecules with translational energy less than the activation energy of freezing will arrive at a site in the ice lattice with kinetic energy low enough to freeze after being accelerated by conversion of potential energy to kinetic. The energy of freezing is then the integral of the product of this energy deficiency and the frequency. In this scheme, a water molecule in the ice at the liquid interface with translational energy equal to the activation energy of melting would have the same total energy as a water molecule in the liquid at the ice interface with translational energy equal to the activation energy of freezing. The resonance between the two would be similar to a pendulum swinging. At 0°C the energies of freezing and of melting were calculated to be approximately 50 cal mol^{-1} [1] — the energy of recrystallization.

The frequency distribution shifts downward on the energy scale as the temperature decreases, and the energy of freezing becomes greater than the energy of melting. Because freezing is a process catalyzed by the ice–liquid interface, supercooling of more than 10°C commonly occurs in plants before some heterogeneous nucleus initiates the process. This results in a high intensity nonequilibrium freeze. Because thermal diffusion can exceed the rate of freezing, rapid cooling also causes measurable supercooling during ice crystal growth. Although the degree of supercooling is slight, the total energy of nonequilibrium crystal growth can be high when many water molecules are involved [3,4]. Cycles of thawing and refreezing accentuate this effect. Cell wall mucilages of hardy cultivars inhibit growth of ice crystals and control freezing processes that can injure the structure of the lower crown, especially during mid-winter thaw–freeze cycles [4–6].

Freezing can occur as an equilibrium process if solutes are present which concentrate in the liquid as freezing progresses. This well-known colligative effect reduces the frequency of water molecules in the liquid at the interface which maintains a balance between freezing and melting, but this osmotic stress develops slowly as a function of decreasing temperature.

Another means by which these energies can balance occurs when ice crystal growth approaches a hydrated substance. The energy of hydration decreases the activation energy of melting at the interface [2]. This results in a decrease in latent heat, which is a measure of adhesion between the hydrated substance and ice through the interfacial liquid for which they compete. The decrease in latent heat of freezing water associated with cellulose was measured with a Calvet microcalorimeter [2]. The measured change in latent heat as a function of decreasing temperature approximately equalled the theoretical value — the entropy of liquid water [7].

Relaxation of interfacial tension by release of sugar into periplasmic liquid from fructan hydrolyzed in the cell vacuole would convert adhesive

to osmotic stress. Significant differences between winter cereal cultivars occur as winter progresses, especially with respect to fructan accumulation during mild weather and its breakdown to sucrose under stress [8,9]. Biochemical changes induced by freezing have been described previously [10–13].

The objective of the current research involved calorimetric monitoring of thermal effects in frozen plant crowns at −3°C to see if the hydrolysis of fructan to sugar could be detected as the corresponding melt of ice required to relax adhesions.

EXPERIMENTAL

Plants of 'Rosen' rye (*Secale cereale* L.) were grown from seeds in sand with Hoagland's nutrient solution at 15°C with light intensity of 175 μmol m^{-2} s^{-1} for five weeks. Then they were hardened at 2°C with continuous light at either 30 (low light) or 115 (high light) μmol m^{-2} s^{-1} for three weeks.

Crowns (1 cm length of tissue in leaf sheathes above the junction with roots) were analyzed for changes in soluble carbohydrates and for thermal activity while frozen and kept at −3°C. Crowns were tested for injury from 1 week in tubes at −3°C by observing new growth at 20°C. All crowns produced new leaf tissue at a rate corresponding to that of the control plants. Injury from freezing or storage at −3°C was not apparent.

Carbohydrate analysis

After each specified −3°C freeze treatment, crown tissues were sliced and transferred to 70°C absolute ethanol. They were kept at 65°C for 20 min to inactivate enzymes. The alcohol solution was separated from the solid matter, which was the ground in distilled water. Five water extracts were combined with the initial ethanol extract and evaporated to dryness under vacuum.

Soluble carbohydrates were fractionated in water by liquid chromatography (HPLC) through a Bio-Rad aminex HPX-87P column at 85°C. Fructan, the principal storage carbohydrate in vegetative tissue of winter cereal plants [14–16], was characterized by HPLC of sugar resulting from complete acid hydrolysis of the fructan fraction [8,9]. This fructan yielded an average of 11 fructose molecules to one glucose molecule.

Thermal analysis

Crowns of rye were studied in a Calvet microcalorimeter. This instrument has 4 crucibles, each with a volume of 12.5 cm^3. Their thermopiles are electrically connected as two opposing pairs. The Seebeck circuit

TABLE 1

Decrease of fructan [a] (mg per g tissue (dry weight)) in frozen crowns at −3°C

Time (days)	Hardened in	
	Low light [b]	High light [c]
1	21	12
2	29	26
4	55	44

[a] The decreases in fructan values were obtained from the intercepts of 230 and 610 mg fructan per g crown tissue with the mean log(sugar/fructan) vs. sugar plus fructan plot for each time interval. These plots were based on 28, 19, 18 and 10 data points respectively for 0, 1, 2, and 4 days frozen at −3°C; manuscript in preparation with data analyzed as in ref. 9.

[b] 230 mg fructan per g crown tissue (dry weight). Average of 19 replicate hardened plant samples.

[c] 610 mg fructan per g crown tissue (dry weight). Average of 10 replicate hardened plant samples.

required 18.5 μW of crucible heating per μV displacement from the baseline. The temperature of the calorimeter core was stabilized at −3.0°C in a refrigerated chamber at −10°C before introducing plant tissue.

Four grams of crowns (wet weight) were placed in one crucible of a pair and frozen at −3°C before insertion into the thermopile. The corresponding crucible of the other pair contained the same weight of similar crowns, but were heated to 80°C for 20 min before freezing at −3°C. The most stable data was obtained in the morning of each day, and measurements were recorded for one week in each test. Then the experiment was repeated, but with the live and heatkilled samples in opposite crucibles from the first test. The entire experiment was repeated during the next two weeks with crowns from plants hardened at the higher light intensity.

RESULTS

Carbohydrate analysis

A continuous decrease in fructan occurs in frozen plant crowns (Table 1). The average decrease in fructan during the first five days at −3°C was 16.4 for low light and 12.0 for high light crowns per gram (dry weight). The molal concentration of a solution at equilibrium with ice of −3°C equal 0.26 g fructose or 0.46 g sucrose per g liquid water. Therefore, hydrolysis of fructan to fructose and sucrose (10:1) could melt 59 mg of ice in the low light crowns and 43 mg of ice in the high light crowns per day per g of tissue.

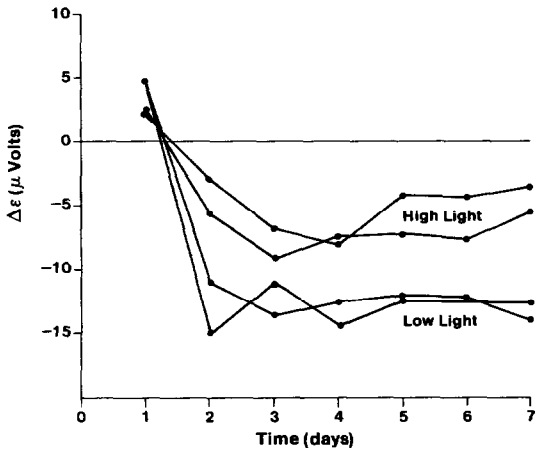

Fig. 1. Voltage generated in the thermopile of a Calvet microcalorimeter per g of frozen crown tissue (dry weight) held at −3°C. The 496 thermocouples in each circuit required 18.5 μW of heat into the crucible per μV output.

Thermal analysis

The voltage generated by thermal activity in the living tissue is presented in Fig. 1. No thermal effect was found in the crucibles that contained killed tissue. Although the tissue was frozen in the crucible at −3°C before placing it in the thermopile, a stable cooling rate did not develop until after the second day.

Crowns from plants hardened in low light gave a mean value of −12.6 μV per g tissue (dry weight) from the third to the fifth day. Crowns from plants hardened in high light gave a mean value of −7.2 μV per g tissue (dry weight) for the same period. This equals 4.82 vs. 2.76 cal per day comparing low light to high light crowns, or melting of 60.6 vs. 34.6 mg of ice per day per g of living tissue.

DISCUSSION

The rate of heat uptake by frozen crown corresponds with the rate of fructan hydrolysis to sugar in terms of the melting required to produce a solution with a freezing point of −3°C. The difference between the plants hardened under low light and those hardened under high light may involve the proportion of cell volume occupied by fructan. Their cell size is similar because initial growth occurred in the same environment. During hardening the plants under higher light not only produce more fructan than those under low light, but also develop a lower sugar content. Because fructan occupies more of the cell volume, the liquid volume diminishes, and a decrease in solute content would be required to maintain normal turgor

pressure. A corresponding decrease occurred in moisture content of the hardened tissue (82% in crowns of plants hardened under low light vs. 77% under high light). Hydrolysis of fructan to intercellular sugar in frozen plants causes relaxation of interfacial tension which converts freeze stress from adhesive to osmotic [1,2,7]. The intercellular solutes are being extracted by a perfusion technique to quantitatively characterize the changes induced by freezing [17]. Hydrolysis of fructan to intracellular sugar would increase turgor pressure and cell volume in frozen plants, which has also been described as a limiting aspect of plant survival [18,19].

Freeze stresses tend to occur sequentially because the different energies develop at different rates as a function of decreasing temperature. Cycles of warming and cooling in frozen tissues increase the probability of disruption. For example, solutes released from protoplasts at colder temperatures where adhesive stress could be damaging interfere with freeze inhibitors which might be needed as ice crystals again grow after a partial thaw. Hardy plants quickly resorb sugars as the temperature increases. Responsive adaptation to changing stress may be difficult to attain in foods. However, the hardiest winter cereal plants continuously adapt to changes in the winter environment, and many of these adaptive mechanisms do not depend on induction of a genetic system. Freeze inhibition, relaxation of adhesion, and osmotic adjustment are induced by specific forms of stress in plants which produced the protective mechanism at a preceding stage of development.

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