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Changes in Osmotic Pressure and Mucilage during Low-Temperature Acclimation of *Opuntia ficus-indica*[1]

Guillermo Goldstein and Park S. Nobel*

Department of Biology and Laboratory of Biomedical and Environmental Sciences, University of California, Los Angeles, California 90024

ABSTRACT

***Opuntia ficus-indica*, a Crassulacean acid metabolism plant cultivated for its fruits and cladodes, was used to examine chemical and physiological events accompanying low-temperature acclimation. Changes in osmotic pressure, water content, low molecular weight solutes, and extracellular mucilage were monitored in the photosynthetic chlorenchyma and the water-storage parenchyma when plants maintained at day/night air temperatures of 30/20°C were shifted to 10/0°C. An increase in osmotic pressure of 0.13 megapascal occurred after 13 days at 10/0°C. Synthesis of glucose, fructose, and glycerol accounted for most of the observed increase in osmotic pressure during the low-temperature acclimation. Extracellular mucilage and the relative apoplastic water content increased by 24 and 10%, respectively, during exposure to low temperatures. These increases apparently favor the extracellular nucleation of ice closer to the equilibrium freezing temperature for plants at 10/0°C, which could make the cellular dehydration more gradual and less damaging. Nuclear magnetic resonance studies helped elucidate the cellular processes during ice formation, such as those revealed by changes in the relaxation times of two water fractions in the chlorenchyma. The latter results suggested a restricted mobility of intracellular water and an increased mobility of extracellular water for plants at 10/0°C compared with those at 30/20°C. Increased mobility of extracellular water could facilitate extracellular ice growth and thus delay the potentially lethal intracellular freezing during low-temperature acclimation.**

Cacti, succulent plants utilizing CAM, generally are native to hot, arid regions where severe freezing seldom occurs (5, 22). Indeed, most cacti will not survive an exposure to −10°C for even a few hours (22). Many species, however, grow at high elevations and latitudes and are therefore exposed to appreciable freezing. *Oroya peruviana* and *Tephrocactus floccosus* occur at 4700 m in tropical alpine habitats in Peru, where summer nighttime temperatures can be −8°C (14), and *Opuntia fragilis* occurs up to 58°N in Canada, where winter temperatures can be −40°C (5, 22). Certain species, such as *Coryphantha vivipara* and *Pediocactus simpsonii,* can tolerate −20°C for 1 h (21) and *Opuntia polyacantha* can tolerate −24°C for 1 h (26).

The tolerance of freezing temperatures by cacti occurring in cold environments significantly increases upon exposure to low temperatures (22, 25). For instance, the low-temperature tolerance of *C. vivipara,* which occurs in the southern prairie provinces of Canada (50°N), decreases from −15 to −22°C as the ambient air temperature is lowered from 30 to −10°C in 10°C steps at weekly intervals (20). These cacti lack the capacity for substantial supercooling (cooling below the equilibrium freezing temperature without freezing) and survive by tolerating extracellular ice formation. Such ice formation in the extracellular spaces leads to a progressive distillation of cellular water, with intracellular dehydration eventually causing irreversible damage (20). Low-temperature tolerance and acclimation have since been examined for 22 other species of cacti (22, 23, 25). These studies have demonstrated that species with the greatest low-temperature tolerance, such as *C. vivipara* and *Opuntia humifusa,* exhibit substantial acclimation. The cellular events that underlie low-temperature acclimation, however, are not well understood.

Cellular events accompanying low-temperature acclimation have been extensively studied for C_3 plants (16, 28). For instance, restructuring of cell membranes can occur (31), including the incorporation of newly synthesized phospholipids (30). Also, increases in osmotic pressure as a consequence of a gradual water stress tend to enhance low-temperature tolerance (16). The synthesis of various low mol wt solutes, such as glucose, fructose, and sorbitol, is also a common feature of low-temperature acclimation (28). High cellular water content in CAM plants, such as cacti, may lead to different events accompanying the low-temperature acclimation compared with C_3 and C_4 plants. For example, transpiration rates of cacti may be too low to permit a substantial increase in osmotic pressure of the cells by dehydration during low-temperature acclimation.

The present study examined chemical and physiological changes during acclimation to low temperatures for *Opuntia ficus-indica,* a cactus extensively cultivated for its fruits and cladodes (22, 27). The tolerance to freezing temperatures for this species, whose range of cultivation is limited by low temperatures, increases 3°C in response to decreasing day/night temperatures, such that acclimated plants can tolerate about −10°C (23). Because mucilage, a highly branched polysaccharide produced by specialized cells and associated with cell walls, is prevalent extracellularly in the cladodes of *O. ficus-indica* (9, 33), its role in the freezing process was analyzed. Special attention was given to water relations parameters such as osmotic pressure, which substantially increases during the winter for cacti that tolerate low tempera-

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tures (15, 17). Also investigated were possible increases in concentration of low mol wt solutes that may act as cryoprotectants as well as help retain liquid water inside the cells. NMR studies were performed during the process of ice formation to help elucidate cellular changes during low-temperature acclimation.

MATERIALS AND METHODS

Plant Material

Cladodes of *Opuntia ficus-indica* (L.) Miller (Cactaceae) were obtained from a commercial plantation in Gilroy, CA, and were planted in 4-L pots containing 6 kg of sandy soil. The plants were maintained in a glasshouse at the University of California, Los Angeles, with day/night air temperatures averaging 26/16°C, day/night relative humidities averaging 40/60%, and a daily PPFD on a horizontal surface averaging 30 mol photons m^{-2} day^{-1} from February to December 1990. The air inside the glasshouse was charcoal filtered and the CO_2 levels were about 360 μmol mol^{-1}. The plants were irrigated twice per week with distilled water and once every 2 weeks with a 0.25-strength Hoagland solution No. 1 supplemented with micronutrients (22). Soil water potential in the root zone was maintained above −0.3 MPa, as determined with Wescor PCT 55-05 soil thermocouple psychrometers.

Between September and December 1990, intact plants were transferred to a growth chamber with 30/20°C day/night air temperatures. The PPFD on a horizontal surface at the top of the plants was 800 μmol m^{-2} s^{-1}, provided daily for 12 h by high-pressure sodium-vapor lamps, leading to a similar total daily PPFD on the cladode surfaces as in the glasshouse. The plants were maintained under the new growth conditions for at least 2 weeks before measurements. To induce low-temperature acclimation, the day/night air temperatures were reduced to 10/0°C.

Water Relations Parameters

The π[2] and water content were measured for tissue samples obtained from mature cladodes of *O. ficus-indica*. Cylindrical cores through the cladodes were removed with a cork borer 13 mm in diameter. The cores were immediately wrapped with Parafilm and sealed in plastic bags to prevent changes in water content before measurements. The chlorenchyma was defined as the outermost 4 to 6 mm of stem tissue inside the epidermis and contained nearly all of the Chl; the remaining tissue was designated the water-storage parenchyma (10–14 mm in thickness). After removing the epidermis, the two tissues were separated from each core with a razor blade and were blotted lightly (to remove sap from cut cells). Mucilage canals and vascular bundles occurred primarily between the chlorenchyma and the water-storage parenchyma and were not included in the samples examined.

The π was determined for cell sap extruded from each tissue using a vise, with the samples squeezed between plastic plates. The osmolality of the extruded sap was determined with a Precision Instrument μOsmette 5004 freezing-point-depression osmometer and converted to π at 20°C using the Van't Hoff relation (24). The water content was determined for 0.3 to 0.4 g tissue samples. Dry weight was determined after drying the samples in a forced-draft oven for 24 h at 60°C. The water:dry matter ratio was expressed as g of liquid water (tissue fresh weight − tissue dry weight) per g of dry sample. ψ isotherms, conventionally called P-V curves, were obtained for excised tissue samples using psychrometric determinations of ψ and RWC calculations; to determine ψ parameters, $1/\psi$ was plotted *versus* 1 − RWC, *i.e.* a type II transformation (24, 34, 35). For instance, the apoplastic water fraction was calculated from the intersection of the linear part of the P-V curve with the abscissa (35, 36). ψ was measured with a Wescor 5500 vapor pressure osmometer calibrated daily, and RWC ([fresh weight − dry weight]/[turgid weight − dry weight]) was estimated using the fresh and turgid weight for a subset of samples undamaged by dehydration (10). To minimize the effect of diel changes in CAM on ψ components (22), all tissue samples were obtained in mid-afternoon.

Mucilage and Other Substances

Mucilage was extracted from the chlorenchyma and the water-storage parenchyma (10, 19). Four grams of tissue were removed with a cork borer (13 mm in diameter), cooled to −80°C, ground in a mortar, and then transferred to boiling ethanol for 5 min to arrest enzymatic activity. After vacuum filtering through Whatman No. 1 paper, the residue was dried at 50°C for 24 h, solubilized for 1 h in 30 mL of distilled water at 50°C, and then vacuum filtered to remove cell wall material and other large debris using a metal sieve (pore size of 70 μm). Mucilage was precipitated with 250 mL of isopropanol, and the precipitate was washed three times in isopropanol followed by air drying. Analysis of sugars characteristic of mucilage (10) and measurement of polypeptides via a micro Kjeldahl technique indicated that at least 93% of the washed precipitate was mucilage. The osmolality of the mucilage dissolved in distilled water was determined with the Wescor 5500 vapor pressure osmometer.

Certain small mol wt substances (sugars and polyhydroxy alcohols) were also analyzed. One gram of the chlorenchyma or the water-storage parenchyma (obtained with the cork borer) was frozen at −80°C, homogenized in a mortar, filtered through a 130-μm nylon mesh, and then centrifuged at 70,000*g* for 1 h. The supernatant fluid was filtered through a 0.4-μm Millipore filter and separated by HPLC using a Waters Chromatography Sugarpak II column and 0.13 mM Ca EDTA buffer. Compounds were quantified using specific standards.

Freezing Point Determinations

To investigate the pattern of subzero temperatures during freezing, 0.1 to 0.2 g of tissue was placed in sealed glass tubes (50 mm long and 5 mm in diameter). The tubes were placed in a Styrofoam box between heat exchangers through which was circulated 45% water/55% ethylene-glycol (v/v), whose temperature was adjusted with a Haake A-82 refrigerated bath. The temperature of the tissue sample in the tubes was monitored at 20-s intervals with copper-constantan thermo-

[2] Abbreviations: π, osmotic pressure; RWC, relative water content; T_1, spin-lattice relaxation time; T_2, spin-spin relaxation time; ψ, water potential; FID, free induction decay; P-V, pressure-volume.

couples (0.26 mm in diameter), which were placed in contact with the tissue. The temperature of the tissue samples was lowered from 20 to −28°C at about 10°C h^{-1}, similar to the rate in other studies (16, 20, 22). Termination of supercooling was indicated by a rapid increase in temperature representing the release of the heat of fusion of water following ice nucleation.

The possible presence of ice nucleation active bacteria in the samples was checked using serial dilutions of homogenized tissue grown on *Pseudomonas* Difco F agar (1). After resuspension in a sterile solution, colonies were detected using the plate-harvesting technique. Very few bacterial colonies occurred, none were identified as *P. syringae,* and no ice nucleation was observed until the temperature of the suspensions was reduced below −9°C.

Freezing patterns were also examined for extracted mucilage. Mucilage was dissolved in different amounts of distilled water; approximately 20 mm^3 of this mucilage solution was drawn into the center of a 100 mm^3 microcapillary, and one of the ends was sealed in a flame (37). Sample temperature was monitored with thermocouples placed in contact with the microcapillaries and was lowered at about 10°C h^{-1}.

NMR Studies

Approximately 0.2 g samples of tissue was lightly blotted and then placed at the bottom of a glass tube (5 mm in diameter). The tube was inserted into a Bruker AM360 pulsed NMR spectrometer with an 89-mm bore and a superconducting magnet producing a magnetic flux density of 8.5 tesla (hence the radio frequency oscillator was at 360 MHz). Temperature was measured with a copper-constantan thermocouple 5 mm below the sample tube (which was not mechanically rotated). Relaxation times, reflecting interactions between water and various cellular components, were measured to determine the water content in various compartments as well as the water mobility.

The longitudinal T_1 was measured using a 180-τ-90 pulse sequence (τ is the time between the radio-frequency pulses in the sequence and the indicated angle is between the average direction of the original proton spins and that induced by the radio frequency pulse; 6, 7). Values of T_1 were determined from the slope of $\ln(A_\infty - A_\tau)$ *versus* τ, where A_τ is the magnetization amplitude of the FID of the water proton signal following the 90° pulse at τ, and A_∞ is the limiting value of A_τ, analysis being performed using Fourier transforms (to a frequency domain). The transverse T_2 was measured by the Carr-Purcell-Meiboom-Gill technique from the slope of ln A_{echo} *versus* t, where A_{echo} is the magnetization amplitude of the water proton signal occurring at time t ($t = 2\ n\tau$, where n is the number of refocusing pulses and τ was 2 ms) after the initial 90° pulse in the 90-τ-180-2τ-180-2τ ... pulse sequence (7). To reach the experimental temperature of 0°C, the tissue samples were cooled in approximately 3°C steps until thermal equilibrium was achieved for each step (up to 30 min). Using the same cooling protocol (3°C steps), the liquid water content of the tissue samples from 20 to −20°C was obtained from the Fourier transform of the FID produced after applying a short (9–11 μs) 90° pulse at different tissue temperatures (2); after applying a Boltzman factor correction (7, 24), the integral of the transformed signal was assumed to be proportional to the liquid water content (the FID for ice is so rapid that it does not interfere with the signal from liquid water).

RESULTS

Water Relations

Tissue π increased for both the chlorenchyma and the water-storage parenchyma of *O. ficus-indica* during acclimation to low temperatures (Fig. 1A). Specifically, π asymptotically increased from 0.59 to 0.72 MPa in the chlorenchyma and from 0.48 to 0.60 MPa in the water-storage parenchyma when plants previously maintained at 30/20°C were exposed to 10/0°C for 13 d. Most of the changes occurred during the first 7 d, and the increase was faster in the chlorenchyma than in the water-storage parenchyma (Fig. 1A). The water content, measured as the water:dry weight ratio, remained virtually constant during acclimation to low temperatures for the chlorenchyma but decreased by 13% for the water-storage parenchyma (Fig. 1B), despite a high and constant soil water potential. Extracellular mucilage, which initially constituted 24 and 17% of tissue dry weight in the chlorenchyma and the water-storage parenchyma, respectively (Fig. 1C), increased by approximately 25% in both tissues after 13 d at 10/0°C.

To examine the causes of π changes during low-temperature

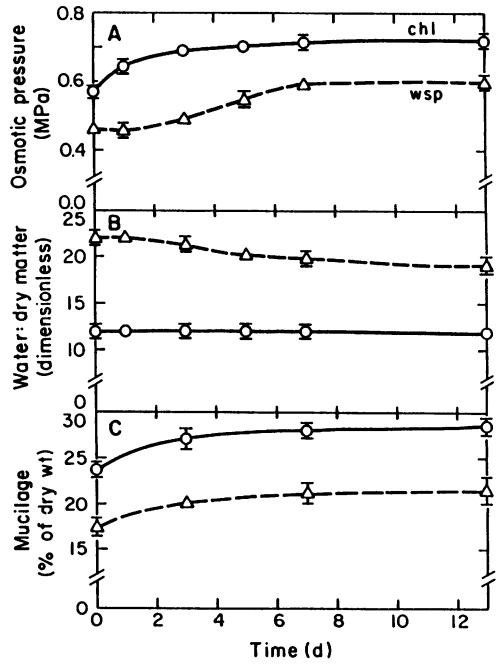

Figure 1. Time courses for the changes in (A) osmotic pressure, (B) the water:dry matter ratio, and (C) mucilage content on a dry weight basis for the chlorenchyma (chl; ○) and the water-storage parenchyma (wsp; △) of *O. ficus-indica* during low-temperature acclimation. The plants were shifted from day/night air temperatures of 30/20°C to 10/0°C at 0 d. Data are means ± 1 SE for two or three measurements on each of two plants (n = 4–6; absence of bar indicates SE smaller than symbol).

acclimation, P-V curves were obtained for the chlorenchyma and the water-storage parenchyma (Fig. 2). π at full hydration (the reciprocal of the intercept of the linear part of the P-V curve with the ordinate) was 0.58 MPa in the chlorenchyma and 0.39 MPa in the water-storage parenchyma for plants at 30/20°C. The apoplastic water fraction based on the P-V curve was very small in both the chlorenchyma and the water-storage parenchyma (about 0.01–0.03) for plants at 30/20°C. After 2 weeks at 10/0°C, π at full hydration in both tissues increased by about 0.10 MPa, and the apoplastic water fraction increased by about 10%.

Consistent with the above changes in the water relations of plants shifted from 30/20°C to 10/0°C, the amounts of glucose, fructose, and glycerol also increased during low-temperature acclimation (Fig. 3). The observed changes were more rapid in the chlorenchyma but larger in the water-storage parenchyma. For example, on a fresh weight basis, fructose increased from 2 to 23 mmol kg^{-1} in the water-storage parenchyma and from 2 to 11 mmol kg^{-1} in the chlorenchyma; the increase was nearly complete in 4 d for the chlorenchyma but was still continuing at 13 d for the water-storage parenchyma (Fig. 3B). The total increase over 13 d for the three organic solutes was 19 mmol kg^{-1} in the chlorenchyma and 47 mmol kg^{-1} in the water-storage parenchyma (Fig. 3).

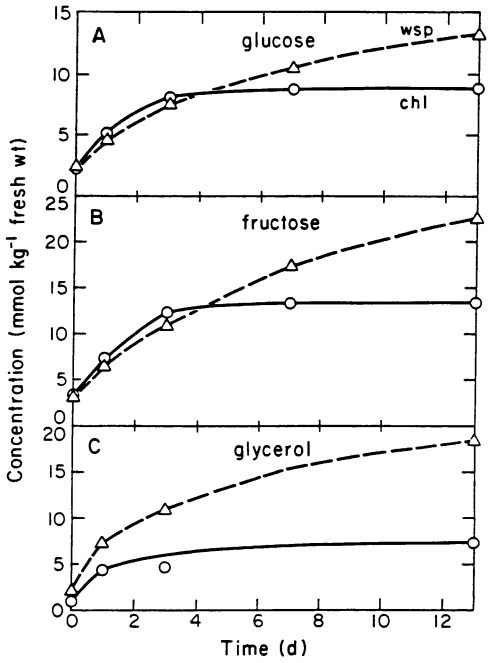

Figure 3. Time courses for the changes in (A) glucose, (B) fructose, and (C) glycerol for the chlorenchyma (chl; ○) and the water-storage parenchyma (wsp; △) of *O. ficus-indica* during low-temperature acclimation. The plants were shifted from day/night air temperatures of 30/20°C to 10/0°C at 0 d. Data are means for single measurements on each of two plants.

Freezing Patterns

As air temperatures were lowered, the temperatures of the chlorenchyma and the water-storage parenchyma decreased below the equilibrium freezing temperature, which was about 0.5°C based on π and the Van't Hoff relation (24). For plants at 30/20°C, exothermic events resulting from ice nucleation caused a sudden increase in temperature at −5.1 ± 1.1°C and −5.5 ± 0.3°C (mean ± 1 SE for $n = 4$) for the chlorenchyma and the water-storage parenchyma, respectively (Fig. 4A, B). In contrast, for plants at 10/0°C, ice nucleation occurred at −2.8 ± 0.5°C (mean ± 1 SE for $n = 4$) for both tissues (Fig. 4C, D). No other major exotherm was subsequently observed as the samples were lowered to −28°C, and all freezing patterns displayed two exothermic phases.

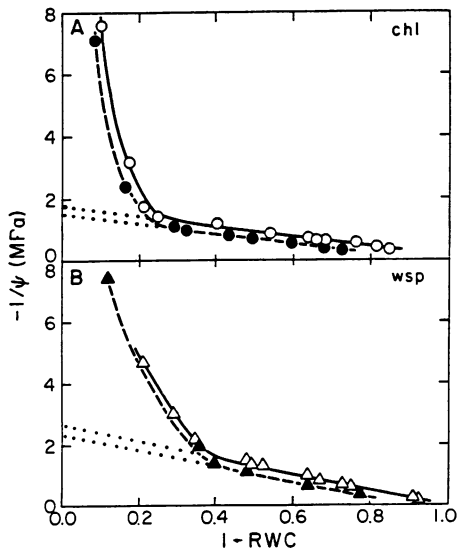

Figure 2. Representative P-V curves for (A) the chlorenchyma (chl; ○, ●) and (B) the water-storage parenchyma (wsp; △, ▲) of *O. ficus-indica* maintained for at least 2 weeks at day/night air temperatures of 30/20°C (○, △) or 10/0°C (●, ▲). ψ and RWC represent the tissue water potential and its relative water content, respectively. The dashed and solid straight lines represent linear regressions, and the dotted lines represent extrapolations of such lines.

Extracted mucilage in distilled water froze at different temperatures, depending on its π (Fig. 5). The ice-nucleation temperatures increased from −12.5 to −7.3°C when π of the mucilage solution increased from 0.0 to 0.5 MPa, most of the changes occurring between 0.0 and 0.3 MPa (0.3 MPa corresponded to 40 mg mucilage/mL). Mucilage from the chlorenchyma and the water-storage parenchyma exhibited similar nucleation temperatures (Fig. 5).

NMR Studies

The relative amount of liquid water in the chlorenchyma and the water-storage parenchyma, as determined by NMR, decreased rapidly below −2.5°C (Fig. 6). The amount of water that remained unfrozen at and below −5°C was less for tissues from plants at 30/20°C than at 10/0°C, especially for the chlorenchyma. At −5°C, the liquid-water content in the chlorenchyma from plants at 30/20°C was 6% compared with 15% after 2 weeks at 10/0°C (Fig. 6A). A similar pattern was observed for the water-storage parenchyma, as the water unfrozen at −5°C was 5% at 30/20°C and 9% at 10/0°C (Fig. 6B).

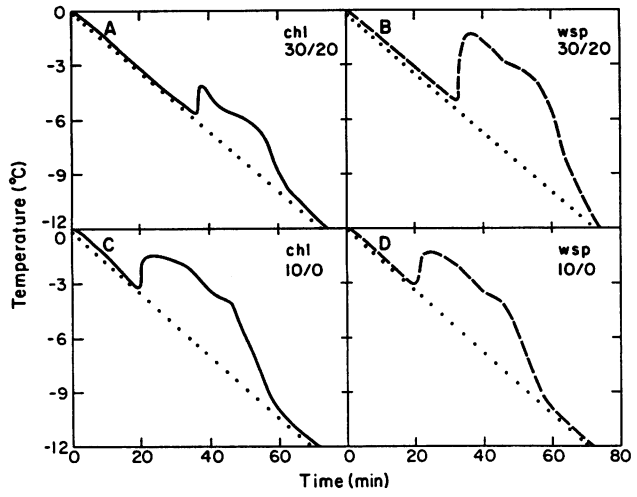

Figure 4. Temperatures during cooling for (A) chlorenchyma (chl) from plants at 30/20°C, (B) water-storage parenchyma (wsp) from plants at 30/20°C, (C) chl at 10/0°C, and (D) wsp at 10/0°C for *O. ficus-indica*. Dotted lines indicate air temperatures. Data are representative of three to four measurements on each of two plants.

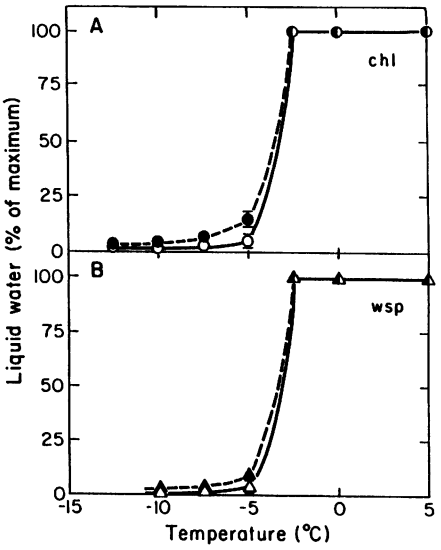

Figure 6. Relative amount of liquid water (percent of maximum) in (A) the chlorenchyma (chl; ○, ●) and (B) the water-storage parenchyma (wsp; △, ▲) of *O. ficus-indica* at various temperatures for plants maintained for at least 2 weeks at day/night air temperatures of 30/20°C, (○, △; —) or 10/0°C (●, ▲; - - -). Data are means ± 1 SE for two measurements on each of three plants; absence of bar indicates SE smaller than symbol.

The magnetization amplitude of the water proton signal for a 180-τ-90 sequence increased as τ was increased (Fig. 7). Although responses for the water-storage parenchyma were fairly linear, indicating a single T_1 (the slope is proportional to $-1/T_1$) so that two water fractions were not distinguishable, a curvilinear relationship occurred for the chlorenchyma. The slope at lower τ was steeper, representing a water fraction with a shorter relaxation time than for longer τ (Fig. 7). The shorter T_1 was lower for the chlorenchyma from plants at 30/20°C than at 10/0°C, whereas T_1 for the water-storage parenchyma was similar for the two day/night air temperatures (Fig. 7).

The T_2 measurements using the echo amplitude also indicated differences between the chlorenchyma and the water-storage parenchyma (Fig. 8). The chlorenchyma again exhibited a curved (biphasic) relationship, indicating the occurrence of at least two water fractions with different relaxation times. The slope (proportional to $-1/T_2$) at lower times was steeper, indicating a shorter T_2 than at longer times; the two values of T_2 tended to be slightly higher for the chlorenchyma from plants at 30/20°C than at 10/0°C. The relationships for the water-storage parenchyma were essentially linear, so two water fractions were not distinguishable, and the slopes were not substantially different for plants at 30/20°C compared with those at 10/0°C (Fig. 8).

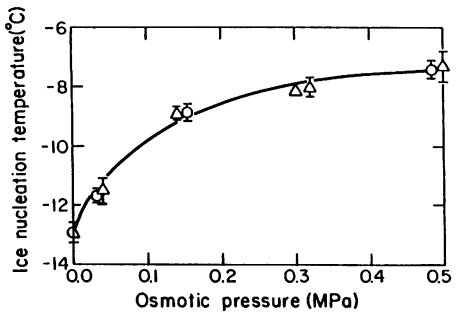

Figure 5. Ice nucleation temperatures for mucilage from the chlorenchyma (○) and the water-storage parenchyma (△) of *O. ficus-indica*. Data are means ± 1 SE for three or four measurements on each of two plants; absence of bar indicates SE smaller than symbol.

Relaxation times were determined for the chlorenchyma from plants at 30/20°C and 10/0°C (Figs. 7, 8) and then corrected for exchanges between the two observed water fractions (Table I). When plants were shifted from 30/20°C to 10/0°C, the corrected T_{1a} (the spin-lattice relaxation time for the slower changing fraction, corrected for water exchange with the other fraction) decreased and the spin-lattice relaxation time for the faster changing fraction (T_{1b}) increased. For the 30/20°C to 10/0°C shift, both T_{2a} (the corrected spin-spin relaxation time for the slower changing fraction) and T_{2b} apparently decreased (Table I). The percentage of water in the two fractions was unchanged by the day/night air temperatures, remaining at 27% for the fraction with the faster relaxation times (P_b). Based on residence times, the time that water spent in the two fractions was 1 to 3 s (Table I).

DISCUSSION

The π and water content of the photosynthetic chlorenchyma and the water-storage parenchyma of *O. ficus-indica* changed when plants were shifted from day/night air temperatures of 30/20°C to 10/0°C. Thirteen days of acclimation to such low temperatures caused π to increase about 0.13 MPa in both the chlorenchyma and the water-storage parenchyma. Most of this increase resulted from an increase in osmotically active solutes, as evidenced by π at full saturation. Although

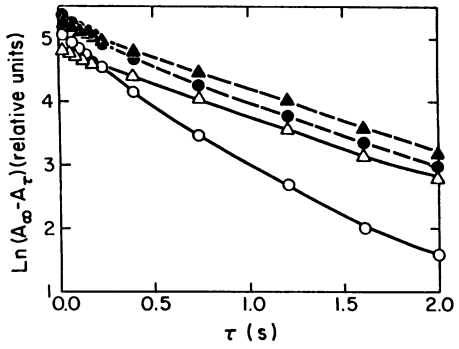

Figure 7. Representative relationships between the magnetization amplitude of the NMR free-induction decay (A_τ) and the time between pulses (τ) for the chlorenchyma (○, ●) and the water-storage parenchyma (△, ▲) of *O. ficus-indica* maintained for at least 2 weeks at day/night air temperatures of 30/20°C (○, △; —) or 10/0°C (●, ▲; - - -). Results were obtained at 0°C.

the water content in the chlorenchyma remained relatively constant during the exposure to 10/0°C, the water-storage parenchyma lost 13% of its water despite wet soil conditions, probably due to the decreased root hydraulic conductivity at low temperatures (18).

According to the Van't Hoff relation (24), increases in fructose, glucose, and glycerol together account for nearly all of the observed π increase during low-temperature acclimation. Specifically, after 13 d at 10/0°C, they represented an increase of 0.13 MPa in the chlorenchyma and 0.10 MPa in the water-storage parenchyma. The increase of such small solutes may enhance tolerance to subfreezing temperatures by osmotically helping retain liquid water inside the cells once freezing is initiated extracellularly. In addition, these solutes may act as cryoprotectants. Polyhydroxy alcohols, such as glycerol and sorbitol, as well as glucose and fructose, increase in C_3 plants during exposure to low temperatures (28). These substances may stabilize the structure of membranes and proteins at subzero temperatures (29), although the mechanism of noncolligative cryoprotection has not yet been fully elucidated.

The amount of liquid, apparently intracellular, water declined rapidly below −2.5°C in both the chlorenchyma and the water-storage parenchyma, but was about twice as high at −5°C for plants at 10/0°C compared with 30/20°C. A small increase in liquid intracellular water during low-temperature acclimation also occurs in several winter cereals (12) but not in *Cornus stolonifera* (6). When water exchange between compartments is rapid, cellular dehydration caused by extracellular ice formation proceeds until the ψ of the protoplast equals that of the extracellular ice. During such equilibrium freezing, the fraction of liquid intracellular water is proportional to 1/T, where T is the temperature in °C and the intercept with the ordinate represents the fraction of bound water (12, 13). This equilibrium behavior has been observed for *Hedera helix* but not for *Hordeum vulgare* (13). For certain citrus species, deviations from ideal freezing behavior were attributed to negative turgor pressures in cells experiencing extracellular ice formation (2). For both the chlorenchyma and the water-storage parenchyma of *O. ficus-indica* at 30/20°C and 10/0°C, the behavior of liquid water during progressive freezing was also apparently nonideal. Further research is necessary to determine whether such nonequilibrium freezing is a general phenomenon for cacti.

Nucleation of ice at temperatures close to the equilibrium freezing temperatures commonly occurs for tissues from species that tolerate substantial intracellular freeze dehydration compared with species that avoid freezing damage by supercooling (8, 11). Although use of small samples may overestimate the extent of supercooling compared with that in intact plants (3), initial nucleation of ice occurred at about −6°C for *O. ficus-indica* at 30/20°C and at about −3°C at 10/0°C. An increase of 3 to 4°C in the ice nucleation temperature during low-temperature acclimation also occurs for *O. humifusa,* a species characterized by appreciable tolerance to subzero temperatures (25). Supercooling ability is often lower for tissues with larger extracellular spaces and, therefore, more apoplastic water (11, 16). A 10% increase in apoplastic water occurred for *O. ficus-indica* during 13 d at 10/0°C, which could reduce supercooling and cause ice nucleation closer to the equilibrium freezing temperature. The exothermic events resulting from ice nucleation exhibited two sequential phases, the first possibly representing freezing of extracellular water and the second the freezing of water distilled from intracellular compartments onto the extracellular ice crystals. The enlargement of the first phase during low-temperature acclimation may correspond to the increase in apoplastic water for *O. ficus-indica* at 10/0°C.

The amount of mucilage in *O. ficus-indica* increased during acclimation to low temperatures, as also occurs for it in response to drought (10). The ice nucleation temperature of mucilage extracted from both the chlorenchyma and the water-storage parenchyma was approximately 5°C higher than for pure water, suggesting that mucilage in the extracellular spaces may act as an ice-nucleating agent. If freezing were induced closer to the equilibrium freezing temperature by a higher extracellular mucilage content (or a higher relative apoplastic water content), the resulting dehydration caused by the net water flux from the cells to the extracellular ice

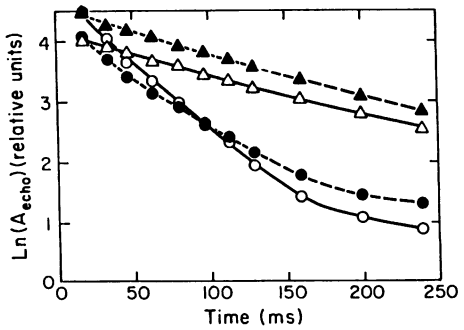

Figure 8. Representative relationships between the magnetization echo amplitude of the NMR free-induction decay (A_{echo}) and the echo time (t) for the chlorenchyma (○, ●) and the water-storage parenchyma (△, ▲) of *O. ficus-indica* maintained for at least 2 weeks at 30/20°C (○, △; —) or 10/0°C (●, ▲; - - -). Results were obtained at 0°C.

Table I. *Spin-lattice (T_1) and Spin-Spin (T_2) Relaxation Times of Chlorenchyma from Plants at Air Temperatures of 30/20°C and 10/0°C*

For *O. ficus-indica* maintained for at least 2 weeks at day/night air temperatures of 30/20°C or 10/0°C, T_1 was calculated from relationships such as in Figure 7 and T_2 from relationships such as in Figure 8. Data are means ± 1 SE for two measurements on each of two plants. The water fraction with the longer relaxation time was denoted by a and the other by b. The relaxation times were corrected to account for water exchange between the two fractions using the principle of detailed balancing (4). The theoretical fraction in state b is designated by P_b ($P_a + P_b = 1$). The mean residence times (time that water occurs in a particular state) in the two states were also determined (4). NMR measurements were performed at 0°C.

Day/Night Air Temperatures	Observed Relaxation Times(s)				Corrected NMR Parameters(s)				P_b	Residence Times(s)	
	T_{1a}	T_{1b}	T_{2a}	T_{2b}	T_{1a}	T_{1b}	T_{2a}	T_{2b}		a	b
30/20°C	0.759 ± 0.007	0.122 ± 0.009	0.081 ± 0.002	0.028 ± 0.001	1.078	0.146	0.051	0.022	0.27	2.1	0.79
10/0°C	0.620 ± 0.030	0.169 ± 0.003	0.076 ± 0.002	0.026 ± 0.001	0.731	0.199	0.040	0.019	0.27	3.3	0.77

crystals would be more gradual as temperature decreases and, hence, should be less damaging. Moreover, the extracellular location of mucilage and its ability to lose substantial amounts of water without experiencing a large decline in water potential (10, 19) could favor water release from the mucilage to the growing extracellular ice crystals and delay cellular water loss.

Two water fractions characterized by short and long relaxation times have also been described in other plant NMR studies (4, 12, 32). The fraction with short relaxation times (low T_1 and T_2) consists of water whose motion is restricted, such as by interactions with macromolecules including cellulose and mucilage, and is generally assumed to be predominantly extracellular. The other fraction has longer relaxation times, hence is more similar to free water, and is predominantly intracellular, presumably vacuolar. Two such fractions of water were detected for the chlorenchyma of *O. ficus-indica,* in which the fraction with the shorter relaxation times accounted for 27% of the tissue water. This is greater than the 5 to 10% of apoplastic water estimated by water potential isotherms, which does not include extracellular (or intracellular) water of hydration. The mean residence times, which represent the average times for the proton spins to leave their water fraction, were of the order of seconds for the chlorenchyma. An even more rapid exchange between the two water fractions apparently occurred for the water-storage parenchyma, because its biphasic behavior was not as readily apparent as for the chlorenchyma.

Both relaxation times of the intracellular water in the chlorenchyma became shorter for plants at 10/0°C day/night temperatures, suggesting greater restriction in the motion of this water during low-temperature acclimation. The restricted mobility is consistent with the decrease in intracellular water and the increase in the osmotic pressure for *O. ficus-indica* acclimated to low temperatures. The T_1 relaxation time of the presumably extracellular water, on the other hand, became longer during acclimation to low temperatures, consistent with the increase in apoplastic water of the chlorenchyma for plants maintained at 10/0°C and with the hypothesized role of extracellular mucilage as a source of water for the growing ice crystals. However, the T_2 relaxation time for the presumably extracellular water did not increase at 10/0°C. Similar responses for T_1 and T_2 of *C. stolonifera* occur during acclimation to low temperatures (6). The increase in T_1 for the extracellular water of *O. ficus-indica* during low-temperature acclimation may represent an increased mobility favoring extracellular growth of ice, which could delay the potentially lethal intracellular freezing. The concomitant decrease in relaxation times for the intracellular water (indicating restricted water mobility) may decrease the rate of water loss through the plasmalemma.

The increase in the ability to tolerate substantial cellular freeze dehydration as ambient temperatures decrease is widespread among cacti native to relatively northern latitudes or high elevations (22). *O. ficus-indica* exhibits an increase in tolerance of 3°C when properly acclimated to low temperatures (23), which could be related to the increases of low mol wt cryoprotectants, an increase in mucilage, and/or increases in the apoplastic water fraction in both the chlorenchyma and the water-storage parenchyma. The large extracellular mucilage content and the large fraction of apoplastic water apparently favor the nucleation of ice close to the equilibrium freezing temperature, which should make the cellular dehydration process more gradual and less damaging. NMR studies indicated that the mobilities of extracellular and intracellular water in the chlorenchyma were affected by acclimation, which may help in understanding the mechanism for avoiding freezing damage. Questions that are unresolved include the apparent nonequilibrium freezing behavior of water in the chlorenchyma and the water-storage parenchyma as well as the relative contributions of the apoplastic water *versus* mucilage for extracellular nucleation of ice in *O. ficus-indica* acclimated to low temperatures.

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LITERATURE CITED

1. **Anderson JA, Ashworth EN** (1985) Ice nucleation in tomato plants. J Am Soc Hortic Sci **110:** 291–296
2. **Anderson JA, Gusta LV, Buchanan DW, Burke MJ** (1983) Freezing of water in citrus leaves. J Am Soc Hortic Sci **108:** 397–400
3. **Ashworth EN, Anderson JA, Davis GA, Lightner GW** (1985) Ice formation in *Prunus persica* under field conditions. J Am Soc Hortic Sci **110:** 322–324
4. **Balla YI, Bakradze NG, Sharimanov YG** (1985) Detection of

two states of water in plant tissues by proton magnetic relaxation. Biophysics (Poland) **30:** 522–528
5. **Benson L** (1982) The Cacti of the United States and Canada. Stanford University Press, Stanford
6. **Burke MJ, Bryant RG, Weiser CJ** (1974) Nuclear magnetic resonance of water in cold acclimating red Osier dogwood stem. Plant Physiol **54:** 392–398
7. **Farrar TC, Becker ED** (1971) Pulse and Fourier Transform NMR. Introduction to Theory and Methods. Academic Press, New York
8. **Franks F** (1985) Biophysics and Biochemistry at Low Temperatures. Cambridge University Press, Cambridge
9. **Gibson AC, Nobel PS** (1986) The Cactus Primer. Harvard University Press, Cambridge, MA
10. **Goldstein G, Andrade JL, Nobel PS** (1991) Differences in water relations parameters for the chlorenchyma and parenchyma of *Opuntia ficus-indica* under wet versus dry conditions. Aust J Plant Physiol **18:** 95–107
11. **Goldstein G, Rada F, Azocar A** (1985) Cold hardiness and supercooling along an altitudinal gradient in Andean giant rosette species. Oecologia **68:** 147–152
12. **Gusta LV, Burke MJ, Kapoor AC** (1975) Determination of unfrozen water in winter cereals at subfreezing temperatures. Plant Physiol **56:** 707–709
13. **Hansen J, Beck E** (1988) Evidence for ideal and non-ideal equilibrium freezing of leaf water in frosthardy ivy (*Hedera helix*) and winter barley (*Hordeum vulgare*). Bot Acta **101:** 76–82
14. **Keeley JE, Keeley SC** (1989) Crassulacean acid metabolism (CAM) in high elevation tropical cactus. Plant Cell Environ **12:** 331–336
15. **Koch KE, Kennedy RA** (1980) Effects of seasonal changes in the midwest on Crassulacean acid metabolism (CAM) in *Opuntia humifusa* Raf. Oecologia **45:** 390–395
16. **Levitt J** (1980) Responses of Plants to Environmental Stresses, Vol 1. Chilling, Freezing, and High Temperature Stresses. Academic Press, New York
17. **Littlejohn RO, Williams GJ Jr** (1983) Diurnal and seasonal variations in activity of Crassulacean acid metabolism and plant water status in a northern latitude population of *Opuntia erinacea*. Oecologia **59:** 83–87
18. **Lopez FB, Nobel PS** (1991) Root hydraulic conductivity of two cactus species in relation to root age, temperature, and soil water cactus. J Exp Bot **42:** 143–149
19. **Morse SR** (1990) Water balance in *Hemizonia luzulifolia:* the role of extracellular polysaccharides. Plant Cell Environ **13:** 39–48
20. **Nobel PS** (1981) Influence of freezing temperatures on a cactus, *Coryphantha vivipara*. Oecologia **48:** 194–198
21. **Nobel PS** (1982) Low-temperature tolerance and cold hardening of cacti. Ecology **63:** 1650–1656
22. **Nobel PS** (1988) Environmental Biology of Agaves and Cacti. Cambridge University Press, New York
23. **Nobel PS** (1990) Low-temperature tolerance and CO_2 uptake for platyopuntias—a laboratory assessment. J Arid Environ **18:** 313–324
24. **Nobel PS** (1991) Physicochemical and Environmental Plant Physiology. Academic Press, San Diego
25. **Nobel PS, Loik ME** (1990) Thermal analysis, cell viability, and CO_2 uptake of a widely distributed North American cactus, *Opuntia humifusa,* at subzero temperatures. Plant Physiol Biochem **28:** 429–436
26. **Rajeshekar C, Gusta LV, Burke MJ** (1979) Membrane structure transition: probable relation to frost damage in hardy herbaceous species. *In* JM Lyon, D Graham, JK Raison, eds, Low Temperature Stress in Crop Plants. Academic Press, New York, pp 255–274
27. **Russell C, Felker P** (1987) Comparative cold-hardiness of *Opuntia* spp. and cvs grown for fruit, vegetable and fodder production. J Hortic Sci **62:** 545–550
28. **Sakai A, Larcher W** (1987) Frost Survival of Plants. Responses and Adaptations to Freezing Stress, Ecological Studies 62. Springer-Verlag, Berlin
29. **Santarius KA** (1982) The mechanism of cryoprotection of biomembrane systems by carbohydrates. *In* PH Li, A Sakai, eds, Plant Cold Hardiness and Freezing Stress, Vol 2. Academic Press, London, pp 475–486
30. **Siminovitch D, Singh J, De la Roche IA** (1975) Studies on membranes in plant cells resistant to extreme freezing. I. Augmentation of phospholipids and membrane substance without changes in unsaturation of fatty acids during hardening of black locust bark. Cryobiology **12:** 144–153
31. **Steponkus PL** (1984) Role of the plasma membrane in freezing injury and cold acclimation. Annu Rev Plant Physiol **35:** 543–584
32. **Stout DG, Steponkus PL, Cotts RM** (1978) Nuclear magnetic resonance relaxation times and plasmalemma water exchange in ivy bark. Plant Physiol **62:** 636–641
33. **Trachtenberg S, Fahn H** (1981) The mucilage cells of *Opuntia ficus-indica* (L.) Mill.—development, ultrastructure and mucilage secretion. Bot Gaz **142:** 206–213
34. **Tyree MT, Jarvis PG** (1982) Water in tissues and cells. *In* O Lange, PS Nobel, CB Osmond, H Ziegler, eds, Physiological Plant Ecology II. Water Relations and Carbon Assimilation. Encyclopedia of Plant Physiology, New Series, Vol 12B. Springer-Verlag, Berlin, pp 35–77
35. **Tyree MT, Richter H** (1982) Alternate methods of analysing water potential isotherms: some cautions and clarifications. II. Curvilinearity in water potential isotherms. Can J Bot **60:** 911–916
36. **Welbaum GE, Meinzer FC** (1990) Compartmentation of solutes and water in developing sugarcane stalk tissue. Plant Physiol **93:** 1147–1153
37. **Zachariassen KE, Hammel HT** (1976) Nucleating agents in the haemolymph of insects tolerant to freezing. Nature **262:** 285–287