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Relationship of Deep Supercooling and Dehydration Resistance to Freezing Injury in Dormant Stem Tissues of 'Starkrimson Delicious' Apple and 'Siberian C' Peach[1]

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Abstract. **Low temperatures (LT) exotherms were found by differential thermal analysis (DTA) at -30°C in 'Siberian C' peach (*Prunus persica* [L.] Batsch) and -39° in 'Starkrimson Delicious' apple (*Malus domestica* Borkh. Nuclear magnetic resonance (NMR) spectrometry of intact stems and isolated bark and wood revealed that the LT exotherm was produced by freezing of deep supercooled water which was detected in the wood but not the bark. Freezing processes of the wood and bark appeared to be independent. In both species, xylem injury occurred at the same temperature as the LT exotherm and was closely, if not causally related to freezing of the supercooled water. Bark injury also occurred at the same temperature as the LT exotherm and may have been caused by dehydration stress or freezing of a small amount of supercooled water which remained undetected by NMR spectrometry. The dehydration resistance of apple wood on desiccation at 70 to 90% relative humidity was greater than that of the peach wood which in turn was greater than that of the bark of both species. The dehydration resistance of apple and peach wood may involve both nonliving and living elements of the wood because pulverizing the tissue destroyed the effect, whereas heat killing only lowered it. Both supercooling and dehydration resistance may be related to microcapillary pore structure which restricts heterogeneous nucleation and sublimation of supercooled water from the ray parenchyma cells.**

Low temperature exotherms (LT) have been detected in the xylem of a large number of woody plants (4, 8, 16), including *Malus* spp. (10) and *Prunus* spp. (14). The temperature at which the exotherm occurred coincided with injury to the ray parenchyma of the xylem. It has been postulated that the exotherm is produced by freezing of a supercooled fraction of water, termed deep supercooling. Water is maintained supercooled within the ray parenchyma by prevention of external seeding (heterogeneous nucleation) and sublimation to ice in adjacent tissue. Injury is caused by intracellular freezing at the temperature at which the supercooled water becomes internally unstable and freezes (homogeneous nucleation) (2).

The relationship of the LT exotherm to bark injury (cambium, phloem, cortex and periderm) is not well understood. In apple, bark injury is coincident with the LT exotherm depending on season and freezing rate (10). In peach, the relationship of the LT exotherm to both wood and bark injury is unknown. Peach is much less hardy than apple. The wood and bark of peach rarely survive below -30°C compared to -40° for apple (13). One purpose of this study was to determine the relationship of freezing patterns in the bark and wood of peach and apple to freezing injury by using differential thermal analysis (DTA) and nuclear magnetic resonance (NMR) spectroscopy. A second purpose was to determine the nature of the barriers which prevent seeding and loss of the deep supercooled water by studying the effects of controlled desiccation.

Materials and Methods

Plant materials. One-year old shoots of 'Siberian C' peach and 'Starkrimson Delicious' apple were collected from mature trees growing outdoors in the orchard of Research Station, Harrow, Ontario, Canada. Samples were sent to the Crop Development Center, University of Saskatchewan, Saskatoon. The samples were not allowed to warm up above 0°C during transit (6 hr). On arrival at Saskatoon, twigs were wrapped with wet paper towels and stored in polyethylene bags at -5° until used. At Harrow samples for DTA and desiccation studies were also stored at -5° until used. The freezing tests were done February, 1978 and 1979 and desiccation studies were done in February, 1979 and 1981.

DTA analysis. DTA analysis was performed on 2 cm twig sections in which the bark and xylem were intact. The method used was as described previously by Quamme et al. (10). The differential temperature was recorded between a sample and a dried reference which were both frozen in an aluminum block at -20°C/hr. DTA analysis was determined on 4 samples of each species.

Tissue injury. Twig pieces 4 cm in length cut from 'Siberian C' peach and 'Starkrimson Delicious' apple were subjected to a temperature decline of 5°C every 12 hr within the range of -5° to -60°.

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Ten twig pieces of each species were removed at 5° intervals and thawed at 0° in air. The twig pieces were incubated for 5 days in a water saturated atmosphere at 22° and then were sectioned with a razor blade and rated for injury under a dissecting microscope (50 x). The cortex, phloem-cambium region and xylem were each scored from 0 to 5 for injury, with 5 being the severest injury.

Nuclear magnetic resonance (NMR) spectrometry. Samples 2 cm in length of intact twigs, bark, and wood were cut 12 cm from the shoot apex of 'Starkrimson Delicious' apple and 'Siberian C' peach. After measuring fresh weight, the twig piece was inserted into a 5 mm NMR tube. The quantity of liquid water in the sample during the freezing process was measured with a pulsed NMR spectrometer (Bruker Mini Spec., p. 20) using the initial free induction decay signal of the sample following a 90 degree pulse (1). The water signal was measured 70 μsec after the initial free induction decay to remove the contribution from the solid signal (dry matter and ice). The sample was frozen at a rate of 0.25°C/min from 0 to -55° and then thawed and dried to obtain the dry weight. The signal of dry matter and ice was confirmed to be undetectable with our NMR set up. The amount of unfrozen water which remained at any subfreezing temperature during freezing was calculated according to Burke et al. (1) except that K factor is zero.

The water content was expressed as g water/g dry wt for freezing studies in the component tissues of the stem (bark and wood). But in the study where the amount of freezing in the intact twig was compared to the total amount of freezing in the isolated components, the water content of the component tissues was expressed as a proportion of the dried stem and added together. The dried weight of the stem and its component tissues were determined after the samples were dried in a vacuum oven at 60°C against $CaSO_4$ for 24 hr.

Desiccation studies. To determine the response to desiccation, bark and wood of peach and apple were subjected to a range of relative humidities in chambers at 21°C. The humidity chambers consisted of 1000 ml erylenmeyer flasks into which mixtures of glycerol and water (200 ml) were placed to attain the required humidity (3). Bark and wood were separated from twig pieces 1 cm long and approximately 0.5 in diameter. Samples of each treatment were placed in aluminum foil and then suspended over the solution in plastic cups. The flasks were sealed with a rubber stopper and then covered with 'Parafilm' which was held in place with elastic bands. The moisture content was measured after they reached constant weight (15 days).

In one study, dehydration resistance of bark and wood were compared at relative humidities from 0 to 100%. Six determinations were made at each relative humidity.

In another study, the dehydration resistance of intact, living wood, heat killed wood and pulverized wood of peach and apple twigs was compared at 4 relative humidities, 80, 85, 90 and 95%. Twig pieces were heat killed by autoclaving at 120°C for 5 min. A Wiley mill was used to pulverize the wood samples. The pulverized wood was passed through a 200 mesh screen and re-hydrated to 55 g H_20/g dry wt for apple and 58 g H_20/g dry wt for peach. A sample of re-hydrated wood from each species was then placed in foil bags for drying in the relative humidity chambers. Four determinations were made at each relative humidity.

Results

Relationship of LT exotherms to injury. Spontaneous ice nucleation of the twig pieces occurred between -8 and -10°C for the peach and -10 to -13° for the apple (Fig. 1 a and b). LT exotherms were detected in twig pieces of both peach and apple with the LT exotherm of the peach occurring at a higher initiation temperature (-30 ± 0.9°) than apple (-39 ± 1.8°). Two LT deflections were consistently found on the DTA profile of peach compared to one for the apple.

There was a close relationship between xylem injury and the LT exotherm in both species (Fig. 1 a and b). The temperature range from the initiation of the LT exotherm to its completion coincided closely to the initiation and maximum development of injury. At any particular temperature the xylem was uniformly affected in cross section by freezing injury.

Some injury was observed in the phloem-cambium regions adjacent to the xylem in apple but not in the cortex, whereas in peach all bark tissues were injured. Bark injury in peach twigs was greater and more extensive in cross section than in apple twigs. Injury to the peach bark was greatest near the xylem similar to apple.

Bark injury of both peach and apple was coincident with the LT exotherm. Bark injury in both peach and apple first occurred at the same temperature interval in which the LT exotherm was initiated. Injury to apple bark did not change appreciably after initial injury but in peach it reached a maximum at the same temperature as the LT exotherm was completed.

NMR freezing curves. Ice nucleation occurred between -13° to -13.5°C in the wood and bark of the peach (Fig. 2a), whereas ice

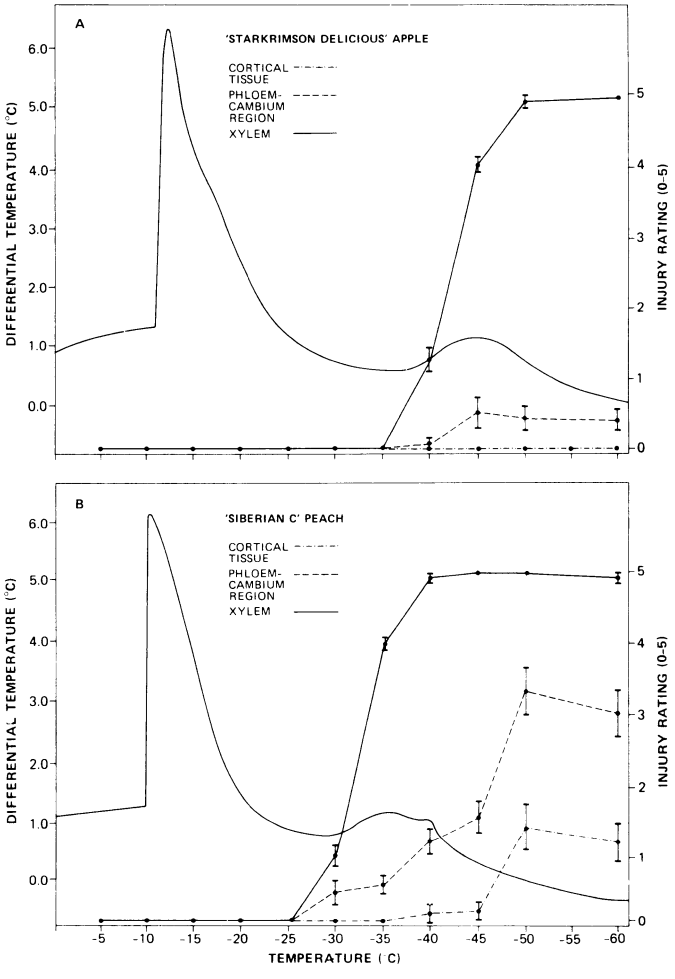

Fig. 1a and b. Relationship of the DTA profile to tissue injury in 'Starkrimson Delicious' apple and 'Siberian C' peach. The injury was scored from 0–5 with 0 indicating no injury and 5 extreme injury.

nucleation occurred at -11° for apple wood and -14.5° for apple bark (Fig. 2b).

After initial freezing, there was only a slight decline in the liquid water of both apple and peach wood until the temperature was lowered to -35°C for apple and -30° for peach (Fig. 2a and b). Thereafter, the liquid water again declined. The second decline corresponded approximately with the initiation of the LT exotherm (Fig. 1a and b). After the second freezing, another plateau was reached at -40° for peach (Fig. 2a and b). The second decline in apple continued to the lowest test temperature (-55°).

The freezing pattern of both apple and peach bark was different from the wood. Once freezing was initiated, water content of the bark declined almost continuously below that of the wood (Fig. 2a and b). The amount of liquid water was a function of temperature and was slightly higher at a given temperature in the apple than peach.

The freezing curve of the intact stem of both apple and peach is almost identical to the curve calculated by adding the liquid water content of the isolated wood and isolated bark together at any given sub-zero temperature (Fig. 3a and b). The observed freezing curve of the intact peach stem was slightly lower than the calculated freezing curve, but the difference was small and within experimental error ($\pm$ 0.02 g/g dry wt). No marked displacement of the calculated curve from the actual curve was observed in either species.

Desiccation studies. The amount of water retained by isolated bark and isolated wood against different relative humidities is shown in Fig. 4. Bark for both species had a higher water content than the wood at a relative humidity of 100%. However, at lower relative humidities the wood of both species had a consistently higher water content than the bark down to a relative humidity of 71%. In the range of 94 to 71% relative humidity, apple wood had a greater water content (termed dehydration resistance) than peach wood. There was greater dehydration resistance at this relative humidity range in wood than in the bark of both species. Below 71% the water content of bark and wood of both species was about the same (not shown).

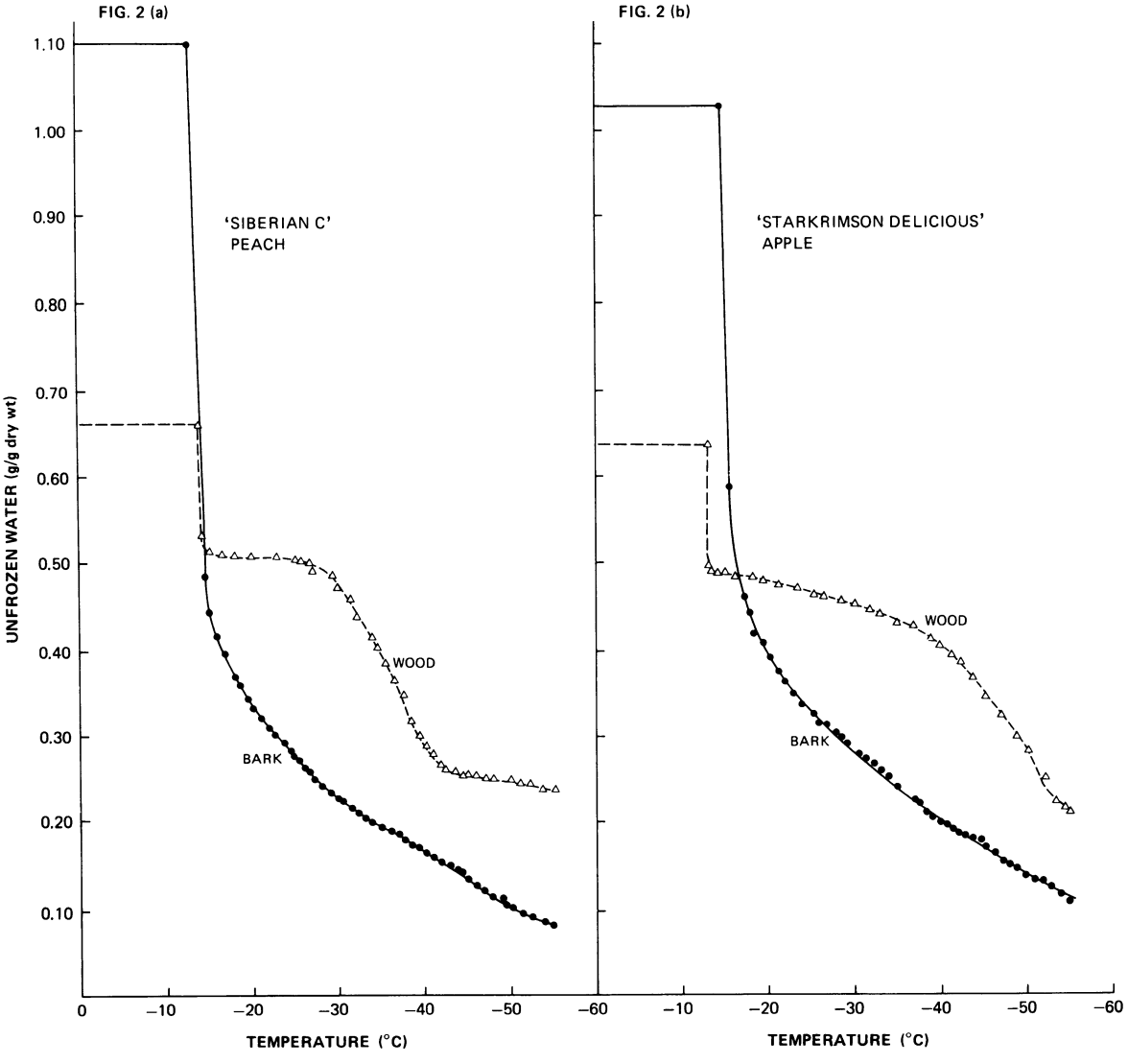

Fig. 2a and b. Typical NMR freezing curve of isolated wood and bark of 'Starkrimson Delicious' and 'Siberian C' peach.

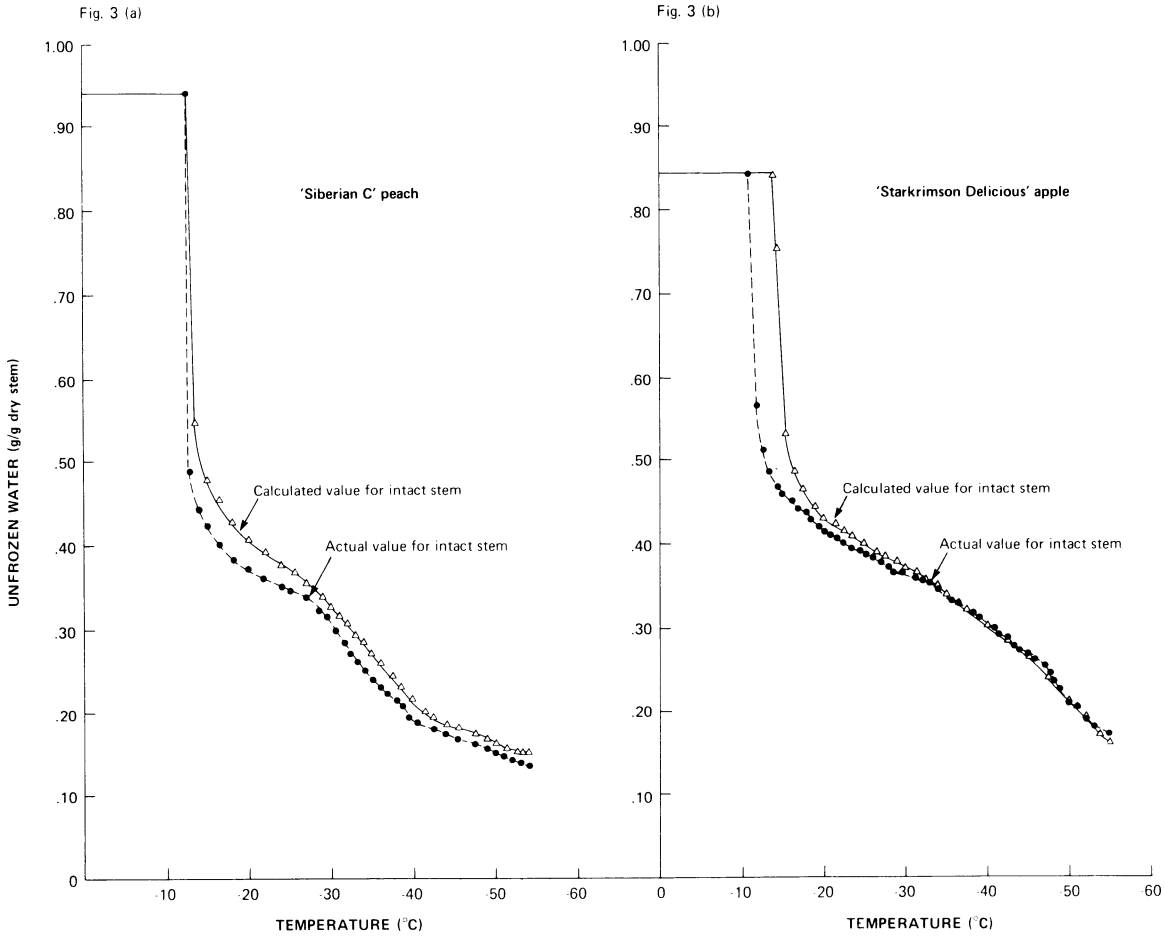

Fig. 3a and b. Comparison of NMR freezing curve of the intact shoot with the curve determined by summing the liquid water content of the isolated wood and bark of 'Starkrimson Delicious' apple and 'Siberian C' peach.

The dehydration resistance of intact, uninjured wood was greater than that of the intact, heat killed wood for both apple and peach (Fig. 5a and b). In turn, the dyhydration resistance of the intact, heat killed wood was greater than that of the pulverized wood for apple at the three highest relative humidities and for peach at the two highest humidities (Fig. 5b and c). The dehydration resistance of intact, uninjured and heat killed wood of apple was greater than that of peach, whereas the dehydration resistance of pulverized wood was similar in both apple and peach (Fig. 5a, b and c).

Discussion

The presence of the LT exotherm in apple wood was reported previously (10), but this is the first report of its presence in peach wood. The double deflection of the LT exotherm in peach is characteristic of tender species (8) and was observed in apple during the early winter season (10).

Separation of the bark from the wood did not greatly displace the NMR freezing curve from that of the intact stem in both peach and apple. On the other hand, full expression of supercooling in the flower buds of peach was disrupted by cutting the flower bud just below the flower (12). The cutting of the flower bud at the base prevented the formation of a dry region during freezing which acted as a barrier to prevent nucleation of the flower bud by external ice.

A sharp decline in the liquid water content as determined by NMR spectrometry coincided with the LT exotherm in wood of apple and peach. This decline is evidence that the LT exotherm was produced by freezing of a deep supercooled water fraction. Xylem injury of both peach and apple was closely, if not causally related to sudden freezing of the supercooled fraction. This supercooling occurs in the pith and xylem parenchyma (11) and appears to result from intracellular freezing (5).

George and Burke (5) were the first to observe that the desorption curve of hickory wood which deep supercools resembled that of solids with an "ink pot" pore structure. The desorption curves of intact, uninjured apple and peach wood also appear to be of the "ink pot" pore type. In solids with the "ink pot" pore structure, high surface tension at the narrow "neck" of the pore reduces water potential and results in a plateau on the desorption curve. George and Burke proposed that the "ink pot" pore structure was responsible for the retention of water in the ray parenchyma cells against the lower vapor pressure over adjacent ice. The narrow neck of the pore may also prevent heterogeneous nucleation by external ice sources. Water is maintained in a stable supercooled state in the cells as long as the temperature remains above the homogeneous nucleation point.

The exact anatomical structure which produces the "ink pot" effect is not known, but it is possible that the "ink pot" pore may involve the cell wall envelope of individual ray parenchyma cells with the interior of the cell acting as the pore cavity and the cell wall pits as the "neck" of the pore. Supercooling does not require the rays to be intact and has been observed to occur as an indepen-

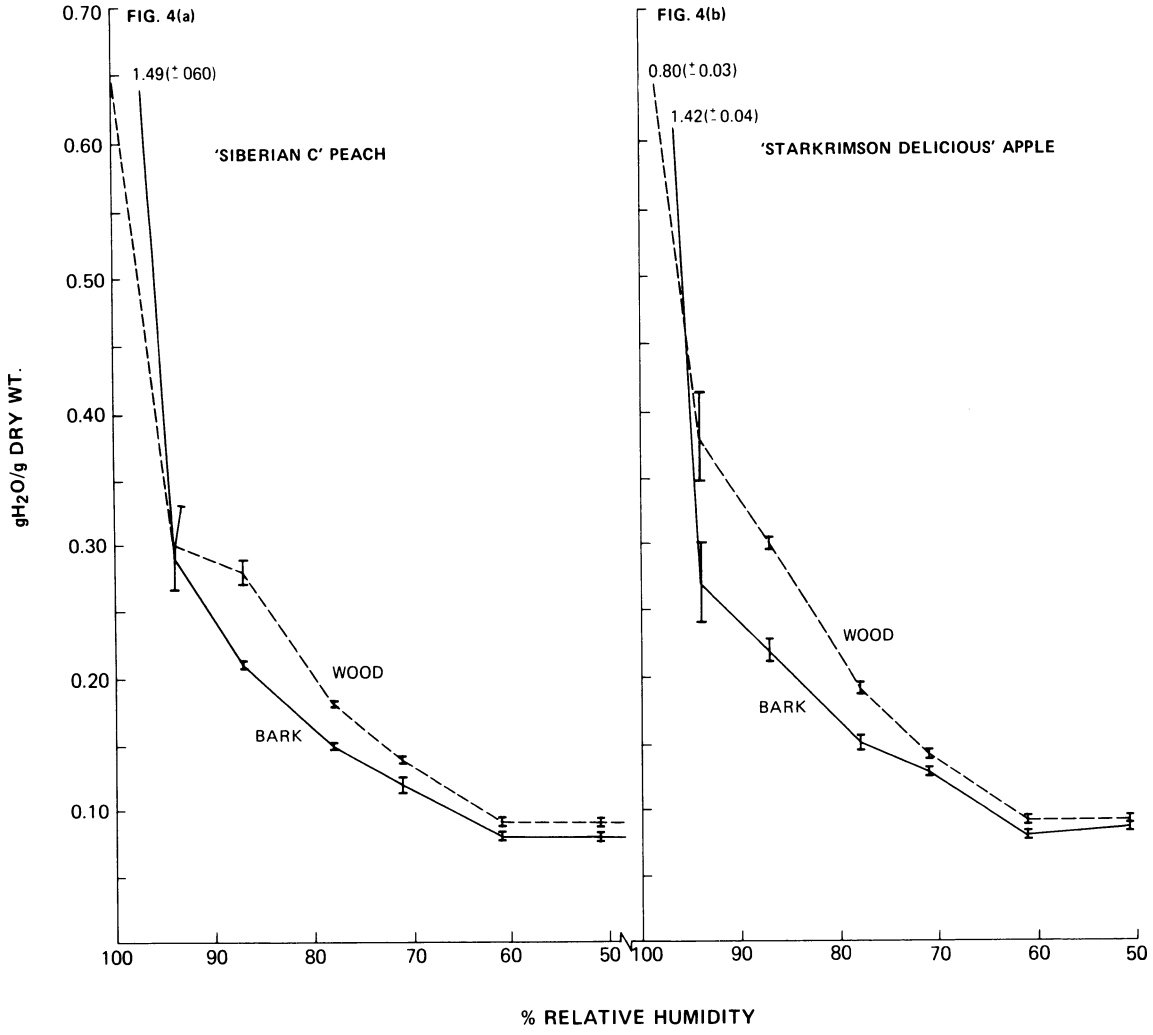

Fig. 4a and b. Water desorption curve of intact bark and wood from 'Starkrimson Delicious' apple and 'Siberian C' peach. The standard errors are presented as lines above and below the point or in brackets.

dent event in a single cell or small groups of cells (7). Presumably, the wood fibers prevent cell collapse. In any event, intact cell wall structure is required because pulverization destroys the dehydration resistance and, as shown in a previous study, supercooling (11).

The presence of the living protoplast may be required for full expression of dehydration resistance and supercooling effects. The dehydration resistance appeared to be partially, but not totally destroyed by heat killing. In previous studies the supercooling temperature was raised by heat killing, drying, chloroform treatment and freezing but supercooling was not eliminated (11).

Apple wood exhibited both a dehydration resistance and greater degree of supercooling than peach. This difference in dehydration resistance may reflect structural differences in the non-living elements of the wood because the difference in response seemed to be present in the heat killed tissue, as well as the uninjured tissue.

After freezing was initiated in the bark, the liquid water content as measured by NMR spectrometry declined continuously to the lowest test temperature (-55°C). The water content reached a low level at the point of injury and declined slowly through the range of injury. Although tissues adjacent to the xylem, i.e., cambium and phloem, were injured at about the same temperature as the LT exotherm, there was no evidence of discontinuities associated with deep supercooling on the bark freezing curve. Peach bark injury was more extensive and severe than apple bark injury in this temperature range, but the freezing curves of apple and peach were similar.

It is possible that bark injury wa induced by desiccation stress during the course of extracellular freezing (9). However, the association of bark injury with the LT exotherm has been observed before in apple (10), in other susceptible *Prunus* spp. (H. A. Quamme, unpublished data) and in azalea (6) and may not be coincidental. It is possible that most of the water had frozen extracellularly in the bark, but a small amount may have been entrapped in certain cells adjacent to the xylem. Intracellular freezing of this small amount of entrapped water may have occurred at the homogeneous nucleation point without producing a definite deflection in the NMR freezing curve. Freezing of small amounts of supercooled water within flower buds (H. A. Quamme, unpublished data) and hickory xylem (5) has been observed to be as damaging as the freezing of large amounts.

Cells which survived to below the LT exotherm may have done so because all of the freezable water was frozen extracellularly. Sakai (15) concluded that apple cortex was able to survive rapid cooling to liquid N_2 temperature (-196°C) after pre-exposure to -

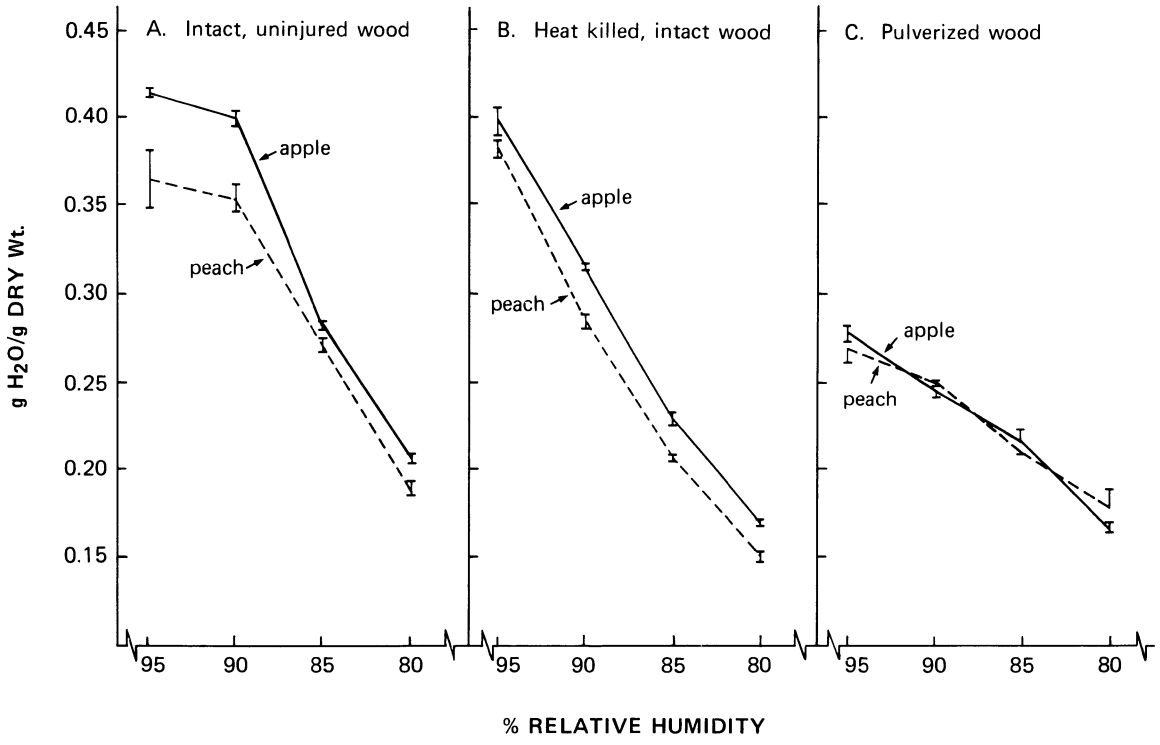

Fig. 5a, b, c. Water desorption in intact, uninjured; intact, heat killed and pulverized wood of 'Starkrimson Delicious' apple and 'Siberian C' peach. Standard errors are presented as lines above and below the point.

30° at slow cooling rates because all the freezable cellular water was frozen extracellularly. The xylem was invariably injured on exposure to temperatures below -30°.

Freezing rate studies conducted on 'Haralson' apple during mid-winter also suggested that bark cells were able to survive below the LT exotherm if slow rates of freezing (0.05°C/min and 0.3°/min) were used. On rapid freezing (1.5°/min) injury occurred to bark cells at about the LT exotherm (10). Evidently, time was required to allow extracellular freezing of the cellular water. Further studies are required, however, to fully elucidate the mechanisms of freezing injury in bark tissues.

Both apple and peach bark had lower dehydration resistance than the wood, which is in agreement with the observation that supercooling is mainly a feature of wood. Presumably, bark cells may not possess the "ink pot" micro capillary structure which prevents water loss and allows deep supercooling to occur.

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