Freezing of Water in Red-Osier Dogwood Stems in Relation to Cold Hardiness

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ABSTRACT

Studies of stem water in red-osier dogwood (Cornus stolonifera Michx.) using nuclear magnetic resonance spectroscopy indicated that most freezing occurs at temperatures above −30°C in cold-hardy and tender stems. Hardy and tender stems had about the same amount of unfrozen water at −40°C (0.28 gram of water per gram dry weight). When hardy stems were slowly cooled below −20°C, the temperature below which little additional freezing occurs, they survived direct immersion in liquid N2 (−196°C). Fully hardy samples not slowly precooled to at least −15°C did not survive direct immersion in liquid N2. The results support the hypothesis that cooling rate is an unimportant factor in tissue survival at and below temperatures where there is little freezable water.

The importance of water in plant injury during freezing has long been discussed. A question often asked is whether injury results directly from intracellular freezing or indirectly via extracellular freezing (13). Several studies have indicated decreases in stem water content concomitant with increasing hardness (8, 9, 14, 15). The stem water content of red-osier dogwood (Cornus stolonifera Michx.) decreases from about 4.9 g water/g dry weight in nonhardy tissue to about 1.75-0.75 g water/g dry weight in hardy plant stems (9). During cooling most of the freezing occurs above −20°C (1). By −20°C the stem water content of both tender and hardy plants is reported to be about 0.25 to 0.30 g water/g dry weight (1). Sakai (11, 12) has shown that several woody species normally killed by quick freezing to −196°C survived such temperatures if they were slowly prechilled to −196°C and subsequently survived temperatures of around −20°C (−15 to −30°C depending on species) before being subjected to −196°C. He suggested that the slow prefreezing gives the plants sufficient time for nearly all of the freezable water to be removed by exosmosis causing cell dehydration and extracellular freezing rather than intracellular freezing and death. There are conflicting reports on the amount of water which remains unfrozen in plants at low temperatures. Typical reports suggest values ranging from no unfrozen water at −196°C (14) to 33% at −120°C (10). Amounts of unfrozen water intermediate between these figures have been found in plants by other investigators over a range of low temperatures from −20 to −110°C (1, 2, 5, 15). In cold collagen fibers, Dehl (3) found that 0.6 g water/g of collagen did not freeze at −50°C. Kuntz and co-workers (6, 7) found that 0.2 to 0.6 g of water/g protein dissolved in solution does not freeze at −35°C. Whether the amount of unfrozen water can be correlated to hardness levels is of some controversy.

Krasavtsev (5), using calorimetric methods, indicated that in a group of species of varying hardness levels, the least hardy had 10 to 23% of water at −60°C while the most hardy species had only 8 to 9%. In earlier studies, Tumanov and Krasavtsev (14) showed that the amount of unfrozen water in shoots dropped continuously as a result of cold acclimation. On the other hand, Burke et al. (1), using NMR spectroscopy, showed that there appeared to be no difference in the amounts of unfreezable water in red-osier dogwood of varying hardness levels at subfreezing temperatures.

The current studies were designed to observe stem water content in red-osier dogwood at various levels of hardness, with particular emphasis on the amounts of unfreezable water at subfreezing temperatures. Water measurements were made using pulse NMR methods. NMR methods have been used in measuring water in biological systems (1-3, 7). Pulse NMR was used here because it is more convenient for the study of water at low temperatures than the continuous wave NMR used by Burke et al. (1) in earlier studies. A more detailed description of the application of pulse NMR to cold hardness is presented elsewhere (2).

MATERIALS AND METHODS

A climatic race of red-osier dogwood (Cornus stolonifera Michx.) native to Dickinson, N.D. was propagated from a single clone as described elsewhere (9), by growing the rooted cuttings in 15-cm pots with a mixture of soil-sand-peat (2:1:1) under 16-hr days and warm temperatures (20/15°C, day/night) for 2 to 3 months. Uniform plants were then transferred to controlled environment chambers and artificially acclimated. Generally plants were acclimated under short days (9 hr) in three consecutive steps: 5 to 6 weeks of warm temperatures (20/15°C, day/night), 3 weeks of cool temperatures (15/5°C, day/night), and 1 week of cool temperatures with a nightly frost (−5°C for 1 hr). Normally, this acclimation scheme produced hardness levels of at least −40°C. Occasionally when harder samples were desired, one of the three steps was extended, usually the frost cycle. Acclimated plants hardened to below −190°C were taken from the field during the winter.

Hardness was evaluated by freezing 2.5-cm stem sections of uniform diameter at a cooling rate of 6 to 8°C/hr. Samples were removed from the freezing chamber, thawed slowly overnight, and then incubated for 7 days at 20°C after which time the cambial tissue was observed for browning as a means of judging viability. Per cent injury was derived from the average of four stems and

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5 Abbreviation: NMR, nuclear magnetic resonance.
was rated on a scale from 1 to 4, corresponding to rating of 2.5 or 50% injury. This correlated well with whole plant and callus regrowth tests conducted in preliminary experiments.

Pulse NMR studies were conducted as described by Burke et al. (2). A 20-MHz Brucker M-20 pulse spectrometer equipped with a programmable temperature controller was used. Briefly the method involved using a series of 90° pulses separated by 6 sec. The free induction decay after the second and each succeeding pulse was monitored less than 6 μsec after the pulse center. The liquid water content, $L_T$, was calculated using equation 1 (2). $T_f^o$ is the free induction decay of the partly frozen sample. $T$ is the free induction decay for the fully thawed sample and $K$ is the fraction of the

$$L_T = \frac{T_f^o - KT}{(1 - K)}$$

total magnetization present from ice when the second and additional pulses are applied. $K$ is dependent on the temperature of the ice and the pulse separation (Fig. 1). The Boltzmann temperature correction was approximated by dividing all NMR signals by the absolute temperature. NMR measurements in stems of varying hardness were recorded under equilibrium freezing conditions. The NMR signal of the oven-dry sample was subtracted from $L_T$ in equation 1 to eliminate nonaqueous contributions to the NMR signal.

RESULTS AND DISCUSSION

Experiments were conducted in which the liquid water was observed over the temperature range of +5 to -70°C in plants of varying hardnesses. Both freezing and thawing characteristics were observed. Representative curves from both a tender plant and a hardy plant are presented in Figure 2. Within experimental limits the freezing and thawing curves were identical. The liquid water is plotted versus temperature ($T$) and $1/T$. The observance of a straight line when liquid water is plotted against $1/T$ has been observed in the freezing of other plants and is expected in the freezing of ideal aqueous solutions as described by Gusta et al. (4). It should be noted that the tender tissue has about seven times the water than is found in the hardy stems. In both cases most of the freezing occurs above -20°C. When one considers that 92% of the total water has frozen in the tender stem while only about 54% of the total water in a hardy stem freezes over a relatively short temperature span, it becomes more clear why the freezing of stem water is so much more damaging in tender tissue. It also points out the importance of the reduction of the amount of water in hardy stems resulting in less damage due to intracellular freezing as compared with tender tissue (8). Tumanov and Krassavtsyn (15), in two different studies on hardy plants, pointed out that rapid thawing from -30 to -60°C in min or even sec failed to cause injury because of the small quantity of water in the plants (23% water per dry weight). The amount of water in the intercellular spaces was not sufficient to cause damage upon thawing. They also showed that the flow of water from hardy cells was almost as rapid as from dead cells (14). Since this rate is more rapid than in nonhardy tissue (5), one can see that cell permeability in addition to water content may be critical in plant survival at subfreezing temperatures.

The differences in liquid water between tender and hardy plants between -30 and -60°C appeared to be insignificant, and indeed, differences in the liquid water at -40°C in plants of varying hardness (Fig. 3) proved to be insignificant. The amount of liquid water at -40°C in this study generally concurs with amounts found in several other investigations (2, 3, 5, 7), and reaffirms the data presented in Figure 2A. For similar red-osier dogwood plants Burke et al. (1) found slightly less liquid water (0.18 g liquid/g dry sample) for fully cold-acclimated plants than we report. This discrepancy is slight and is probably due to differences in the continuous wave versus the pulse NMR methods used in the two studies.

In the same study, Burke et al. (1) found no major differences in the freezing properties of hardy and nonhardy tissue between -10 and -30°C. Figure 4 indicates that slow freezing to -20°C was necessary before hardy dogwood plants could withstand rapid freezing in liquid $N_2$. This concurs with experiments by Sakai (11) which were conducted on a variety of hardy species which could survive liquid $N_2$ temperatures if prefrozen to at least -15°C in midwinter and -30°C in early spring. Sakai (12) suggested that in very hardy twigs the freezable water probably freezes out extracellularly at temperatures of -30°C or above. This suggestion is supported in this study not only by the survival data above, but also by freezing data presented in Figure 4, which show that 87% of the freezable water is frozen above -30°C, while only 60% is frozen above -10°C. As shown above (Fig. 4) the former exhibited no injury while the latter tissue was 100% injured. Fifty per cent injury (the designated killing point) occurred at -15°C at which
Fig. 3. Liquid water content at −40°C (L_40) of seven red-osier dogwood samples of varying hardness levels from −3 to below −190°C. Point 68% of the freezeable water was frozen. This suggests that approximately 70% of the freezeable water must be frozen out of hardy dogwood stems for survival to be expected. Indeed at −20°C with 76% of the freezeable water frozen, the per cent of injured tissue was reduced to 8% (Fig. 4).

These studies show that the rate of cooling is critical above a certain temperature (−15°C in hardy dogwood) because the amount of unfrozen water is sufficient to cause injury at rapid cooling rates. These high rates of cooling have diminishing effects as the amount of freezeable water decreases at lower temperatures. Survival depends on the relative amounts of ice and water present during rapid cooling or on the rate at which water leaves the cells in order to establish thermodynamic equilibrium rather than on the cooling rate of the tissue itself.

LITERATURE CITED


Fig. 4. Per cent cambial injury sustained by pre-freezing fully hardy red-osier dogwood stem sections (surviving −196°C) to successively lower temperatures before placing in liquid N_2 (---). (----): Freezing curve for typical hardy stem sample depicting fraction of total water (L_o) that is liquid (L_r) as represented on the right axis. Stem sections were frozen to −60°C. At 5°C intervals between 0 and −60°C, samples were withdrawn and quickly frozen to −196°C in liquid N_2 for 5 min, quick-warmed to withdrawal temperature where freezing was resumed with the other samples.