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Induction of Frost Hardiness in Stem Cortical Tissues of *Cornus stolonifera* Michx. by Water Stress

I. UNFROZEN WATER IN CORTICAL TISSUES AND WATER STATUS IN PLANTS AND SOIL[1]

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ABSTRACT

Water supply and day length were varied in cold hardiness studies of red osier dogwood plants (*Cornus stolonifera* Michx.). The frost killing temperature, the content and freezing of stem cortical tissue water along with soil moisture content and tension were evaluated. Seven days of water stress in long and short day photoperiod regimes caused a rapid decrease in soil moisture content and plant water potential. During the same period, the frost hardiness increased from −3 to −11 C. Further water stress treatment had little effect. Control plants in short days showed only a gradual decrease in plant water potential and only gradually increased in frost hardiness while control plants in long days were unchanged. Freezing studies using nuclear magnetic resonance showed that increased hardiness in water-stressed plants resulted from both an increased tolerance of freezing and an increased avoidance of freezing, the latter resulting from higher solute concentration in the tissue solutions. The short day controls also showed similar changes; however, the changes were smaller over the 21 days of the study.

Levitt (12) suggests that in some plants freezing tolerance is due to avoidance or tolerance of freeze-induced dehydration of tissue cells during ice formation and growth in the extracellular spaces. One, therefore, expects and finds a similarity of hardiness to plasmolysis, drought, and frost (23). There is ample evidence that frost hardiness and drought hardiness are related. When barley plants are exposed to low temperature to induce frost hardening, they also acquire drought hardiness (13). Evergreens are also more drought hardy in winter (18). Conversely, artificial dehydration of stems of red osier dogwood increases their cold hardiness level (14). In the field during cold acclimation, stem water loss is a natural occurrence even when the roots are in water-soaked soil (17).

In the previous study, Chen *et al.* (5) found that cold hardiness of the stems of red osier dogwood plants (*Cornus stolonifera* Michx.) could be increased by water stress. The increase in cold resistance was correlated with the water saturation deficit of the stem tissues. In this study, we examine the interrelationship among water, the freezing process, and frost killing temperature of water-stressed dogwood plants.

MATERIALS AND METHODS

Preparation of Plants. Red osier dogwood plants (*C. stolonifera* Michx.) were propagated from a single clone (Dickinson, N. D.) by tip cuttings and transplanted into 15-cm pots in a mixture of 2 parts soil-1 sand-1 peat (v/v/v). Plants were watered as needed, grown for 60 days, and trained to two leaders in a lighted greenhouse with a 15-hr photoperiod, and a 20/15 C (day/night) regime. Uniform plants were selected for a 2 × 4 × 2 factorial experiment in a completely randomized design. This included: (*a*) two photoperiods—long day (LD, 14 hr light), and short day (SD, 10 hr light); (*b*) four time periods—3, 7, 14, and 21 days at 20/15 C (day/night) with controlled water supply; and (*c*) two controlled water supply treatments—a control treatment where plants were watered once daily to the point of saturation (*i.e.* 250 ml/plant), and a 30-ml water/day plant treatment. Each potted plant constituted an experimental unit and each treatment was replicated four times in a growth chamber. The growth chamber in both LD or SD regime was maintained at 60% relative humidity, 20/15 C (day/night), and 27 klx light intensity. All plants were watered to the field capacity at the beginning of the test, and each pot was wrapped with a plastic bag to eliminate evaporative losses of water from the soil during the experiment. The measurements were determined after 3, 7, 14, and 21 days of controlled watering treatment. Throughout the 21-day test period, plants were watered between 4:30 and 5:00 PM each day. After the end of each period, soil moisture content, minimum survival temperatures of stem cortical tissues, tissue water contents, amount of unfrozen water at the minimum survival temperature, and plant water potentials were measured.

Determination of Soil Moisture Content and Soil Moisture Tension. At the end of each time period, soil in each pot was collected to determine the fresh weight and then oven-dried at 105 C for dry weight. Soil moisture content was calculated and expressed as g moisture/g dried soil. Soil moisture tension values were obtained from separate pots with plants which were watered to the saturation point and sampled at about 8-hr intervals until the plants completely died. A soil moisture tension curve, *i.e.* the soil moisture content *versus* the soil moisture tension (Fig. 1) was prepared by the method of pressure plates and membrane according to Richards and Wadleigh (21).

Evaluation of Frost Hardiness. The survival of stems subjected to controlled freezing stress (16) was evaluated by the method of visual observation as described by Li *et al.* (15) and by McKenzie *et al.* (17) and expressed as the minimum survival temperature (the lowest test temperature at which stem tissues survived).

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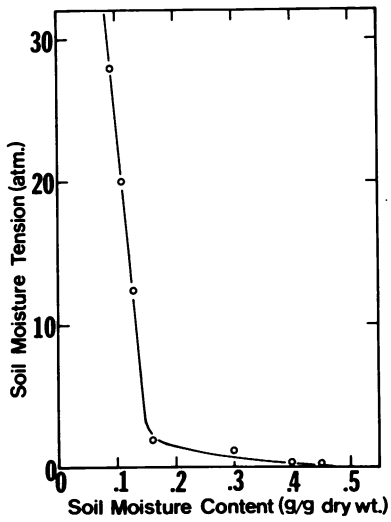

FIG. 1. Soil moisture content and mositure tension relation curve for the soil mixture used in the experiment.

Measurement of Plant Water Potential. A pressure chamber was employed for the measurement of leaf xylem water potential. A leaf at the same position (*i.e.* at the fourth node from bottom) of each plant was detached 5 hr into the photoperiod and immediately inserted into the pressure chamber. The reading was taken at the point when xylem sap started exuding. The technique estimates only the nonosmotic component of xylem water potential, but probably gives the best field estimate of water potential yet developed (2).

Nuclear Magnetic Resonance (NMR) Spectroscopic Study. The epidermis and cortical tissues were peeled from a 22-mm long stem section taken from the middle portion of the fourth internode and inserted into a 5-mm nuclear magnetic resonance tube. After measuring the fresh weight, the tube with tissues was then inserted into a pulsed NMR[4] spectrometer. The amount of liquid water remaining in the partially frozen tissues during the freezing process was measured using the initial free induction decay signal of cortical tissues following a 90 degree pulse (3). The cortical tissues were inoculated at −2 C with a frozen needle and then kept at that temperature until reaching equilibrium. Thereafter, the temperature was lowered in 1 C per step to −15 C and held at each step until no additional freezing could be detected. The temperature was then dropped to −40 C to determine the amount of liquid water remaining. The tissue was thawed and oven-dried at 85 C to obtain the dry weight. The tube with the dry sample was again inserted into the NMR spectrometer to measure the signal caused by the dry matter of tissues. The amount of liquid water remaining in the partially frozen tissues at any subfreezing temperature during the freezing process was calculated as indicated by Burke *et al.* (3). Only one sample for each treatment was measured.

Determination of Saturation Point, Field Capacity, and Permanent Wilting Point of the Soil Mixture. Potted plants were put into a container with water so that 80% of the height of each pot was immersed in water for 24 hr. The soil in the pot was immediately collected and weighed to determine the fresh weight, then oven-dried at 105 C for the dry weight. The soil saturation point was expressed as g moisture/g dried soil. For soil field capacity, the pot with plant was removed from the water after 24 hr immersion, and allowed to drip until leaching stopped. The fresh and dry weight of the soil were then obtained. For permanent wilting point, watering of the potted plants was withheld until the lower leaves wilted. Wilting was regarded as the first permanent wilting point of soil (6). The mean of four samples was obtained for each determination.

RESULTS

Soil Water Status and Changes of Water Potential in Plants by Water Stress. The soil mixture had a saturation point of 0.47 ± 0.02 g H_2O/g dried soil, a field capacity of 0.23 ± 0.01 g H_2O/g dried soil, and a permanent wilting point of 0.11 ± 0.01 g H_2O/g dried soil (Fig. 2). The stressed plants showed incipient wilting after 3 days; the leaves lost turgor progressively from the oldest (*i.e.* from the bottom node) upward until after 7 days, all leaves were wilted in both LD and SD regimes. The leaves of stressed plants regained turgor when the older leaves senesced and died. Thereafter, the stressed plants appeared to adapt to the condition of controlled watering treatment. The changes of moisture content of the soil mixture (Fig. 2) were similar in both LD and SD regimes. The stressed pots lost soil moisture rapidly after 3 days going from about 0.40 to 0.16 g water/g dried soil, and gradually to 0.10 g water/g dried soil after 7 days, then leveling off for the rest of the testing period. Moisture tension curves indicated that the tension increased sharply as the permanent wilting point was approached. Soil moisture content of control pots was maintained at about 0.40 g water/g dried soil throughout the testing period.

Changes of water potential in plants were also similar in both LD and SD regimes except that controls in SD regime decreased in water potential gradually from −5 bars to −11 bars after 21 days, whereas controls in LD regime remained about −5 to −6 bars constantly (Fig. 3). The stressed plants decreased in water potential rapidly from −5 bars to −17 bars after 7 days and then leveled off with little change in both LD and SD regimes.

Frost Hardiness and NMR Study. The water stress treatment increased frost hardiness, decreased tissue water content, and decreased the amount of unfrozen water at the killing temperature in stem cortical tissues (Table I). After 21 days in the SD regime, controls hardened from −3.2 to −6.5 C, decreased tissue water content from 2 to 1.66 (g/g dry matter), and the amount of unfrozen water at the killing temperature changed from 58 to 36%. Controls in the LD did not harden beyond −3 C, did not change in tissue water content (2.02-2.20 g water/g dry matter), and did not change in the amount of unfrozen water at the killing temperature. Plants water stressed with 30 ml water/day·plant survived to −6 C after 3 days and survived to

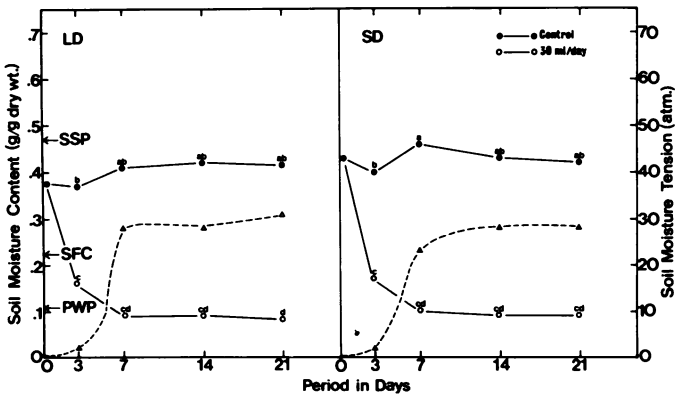

FIG. 2. Soil moisture content of potted plants grown under LD or SD photoperiod regimes after 3, 7, 14, and 21 days of controlled watering treatments. The plants were either watered to saturation point as controls, ●, or watered at a rate of 30 ml/day·plant, ○. The soil moisture tension curve, △, corresponds to the soil moisture curve of potted plants watered at a rate of 30 ml/day·plant. Means followed by the same letter do not differ significantly at the 5% level according to Duncan's Multiple Range Test. SSP is the soil saturation point. SFC is the soil field capacity. PWP is the permanent wilting point.

[4] Abbreviation: NMR: nuclear magnetic resonance.

−11 C after 7 days in both LD and SD regimes. Thereafter, there was little increase in frost hardiness as a result of 14 days additional water stress. Tissue water content of stressed plants dropped rather rapidly from 2.10 to 1.65 g water/g dry matter in LD and to 1.54 g water/g dry matter in SD after 7 days, and then changed very little for prolonged controlled watering treatment. The amount of unfrozen water at the minimum survival temperature in stressed tissues decreased rapidly from 58 to about 40% after 3 days and to 30% after 7 days in both LD and SD regimes. Thereafter, there was only a little decrease in the amount of unfrozen water at the killing temperature.

Cortical tissues of stressed plants tolerated more freezing than control plants (Fig. 4). These plots of unfrozen water (L_T) with the reciprocal of temperature (1/T) gave a simple straight line

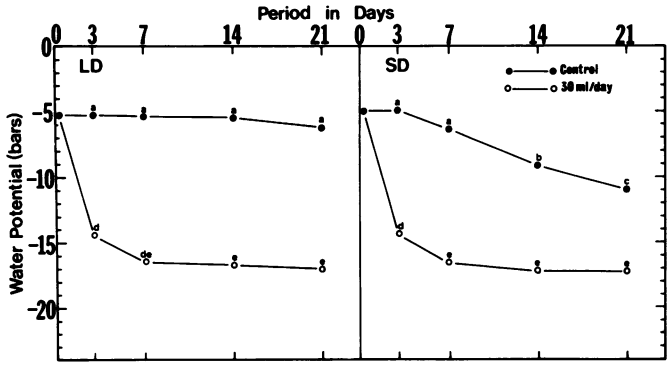

FIG. 3. Xylem water potential of red osier dogwood plants grown under LD or SD photoperiod regimes after 3, 7, 14, and 21 days of controlled watering treatments. The plants were either watered to saturation point as control, ●, or watered at a rate of 30 ml/day·plant, ○. Means followed by the same letter do not differ significantly at the 5% level according to Duncan's Multiple Range Test.

where the slope could be used to estimate the freezing point depression and thus calculate the osmotic potential of the plant tissue (Fig. 4) (7). The slopes of the line for control tissues in LD changed very little while controls in SD increased gradually from −1.29 C after 3 days to −1.43 C after 21 days. The slopes of stressed plants, on the other hand, changed rather sharply and the LD and SD plants increased from −1.32 C to as high as −1.65 C after only 7 days, and then remained unchanged.

DISCUSSION

The stressed dogwood plants showed incipient wilting after 3 days and progressive senescence of leaves from the oldest upward until after 7 days when no further change occurred. This phenomenon is similar to the result obtained with sugar beet during drought treatment (22). Pringsheim (19) found a movement of water from older to younger parts of some plant species (*e.g. Erica, Bryophyllum,* etc.) on wilting, and this protected the younger parts longer and permitted them to develop further. It has also been shown that within a single plant, the younger tissues are always more tolerant to water stress than the older ones, *e.g.* in the plumules of seedlings (20), in the dormant buds of moss (10), and in the younger parts of liverworts (8).

The changes in water potential of these stressed plants in both LD and SD regimes in these controlled growth chamber experiments are closely related to the changes of soil moisture content and also to changes of soil moisture tension (Figs. 2 and 3). Because of the rapid transfer of water from soil to roots and to shoots, it is evident that even in the vicinity of the chloroplasts, which are very close to the shoot environment, the water potential is chiefly controlled by the status of soil moisture (9). The rapid decrease in water potential of stressed plants during the first 3 days, even though the soil moisture tension still remains quite low (about 4 atm), may be due to the increase in the rates of transpiration as soon as soil moisture starts to decrease (1).

Table I. *Nature of Freezing Tolerance in Stem Cortical Tissue of Dogwood Plants by Controlled Watering Treatments.*

Photoperiod	Treatment	Period in Days	Survival Temp (°C)	Tissue Water Content (g/g dry wt.)	% Liquid Water Unfrozen at Survival Temp.
				(n=1)	(n=1)
	30ml/day	0	-3.0a*	2.10	57
		3	-6.0c	1.82	40
		7	-10.5d	1.65	30
		14	-11.0d	1.60	29
		21	-12.0d	1.50	28
Long Day					
(14 hrs light)		0	-3.0a	2.10	57
		3	-3.0a	2.10	56
	Control	7	-3.0a	2.02	57
		14	-3.0a	2.17	58
		21	-3.0a	2.20	57
		0	-3.0a	2.10	57
		3	-6.5c	1.72	38
	30ml/day	7	-10.8d	1.54	30
		14	-11.5d	1.42	29
Short Day		21	-12.2d	1.44	27
(10 hrs light)		0	-3.0a	2.10	57
		3	-3.2a	2.10	54
	Control	7	-4.0ab	1.98	50
		14	-5.0bc	1.78	42
		21	-6.5c	1.66	36

* Within that column, means followed by the same letter do not differ significantly at the 5% level according to Duncan's Multiple Range test.

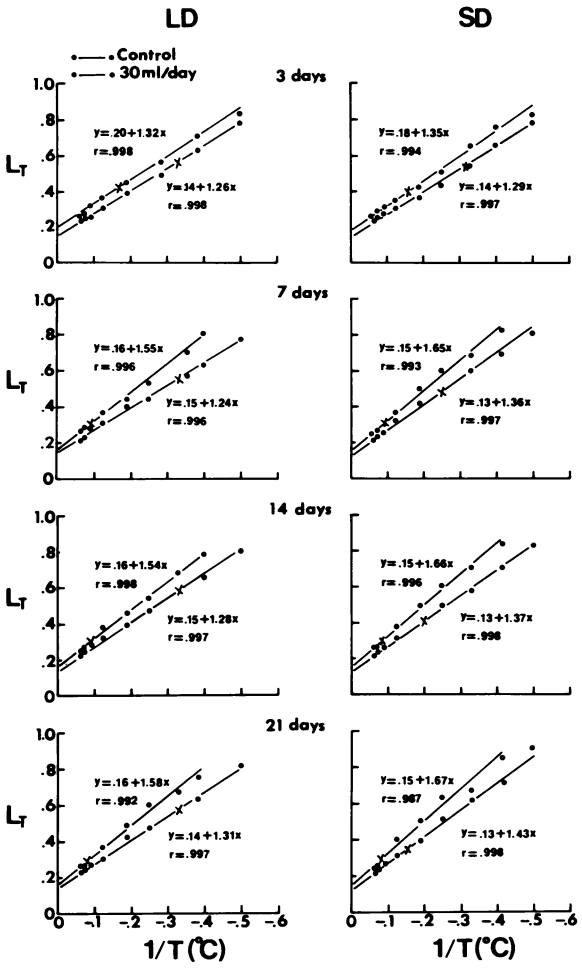

FIG. 4. Plots of fractions of unfrozen water (L_T) against the reciprocal of each corresponding freezing temperature (1/T C) in stem cortical tissues of red osier dogwood plants grown under LD or SD photoperiod regimes after 3, 7, 14, and 21 days of controlled watering treatments. The plants were either watered to saturation point as controls, ●, or watered at a rate of 30 ml/day·plant, O. x on each curve indicates the minimum survival temperature of the tissues.

The gradual decrease in water potential in SD control plants may be due to a decreased stomatal resistance combined with an increased root resistance to water movement as a result of short day-induced cold acclimation (17), even though the plants were in highly saturated soil moisture (Fig. 2).

The freezing curves in Figure 4 indicate that the stressed plants always had greater slopes (*i.e.* melting point depression) than the nonstressed plants, and therefore, at any given subfreezing temperature, the stressed plants had a smaller proportion of their water frozen. From this, one could say the stressed plants are better freezing avoiders. In addition to being better freezing avoiders, the stressed plants were also more tolerant of freezing (Table I). The stressed plants, when compared to the nonstressed, increased tolerance for ice rapidly within the first 7 days of stress treatment. Therefore, the induction of frost hardiness by water stress apparently involved both avoidance and tolerance of tissue ice. Control plants in SD regime also showed an increase avoidance and tolerance of tissue ice (Table I; Fig. 4); however, the effect on the short day controls was less pronounced and continued throughout the 21-day testing period. For the stressed plants, additional water stress after the first 7 days gave very little change in the slope of the freezing curve (melting point depression) (Fig. 4), cold hardiness, or freezing tolerance (Table I). The results may indicate that the induction of frost hardiness by water stress treatments is a rapid protective and adaptive reaction of the plants responding to unfavorable environments. Since the decrease in tissue hydration of dogwood plants during short day conditioning is due at least in part to an increased root resistance to water movement (17), it is likely that the SD plants also acclimate due to a moderate water stress.

The observation that plants increase in avoidance and tolerance of tissue ice is not new. Levitt (11) found this for cabbage which showed melting point lowerings going from 0.81 to 1.26 C and ice tolerance going from 60 to 75% (per cent total water frozen) during cold acclimation. Also, the observation of a simple linear relation between per cent liquid water and the reciprocal of temperature is not new; it has been observed for crowns of winter cereals (7) and leaves of *Solanum* species (4).

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