Environmental and Seasonal Factors Affecting the Frost-induced Stage of Cold Acclimation in *Cornus stolonifera* Michx

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ABSTRACT

Stem tissues of red-osier dogwood (*Cornus stolonifera* Michx.) acclimated from −3 C to −40 or −50 C in eight to ten weeks under a short photoperiod (9 hours) and controlled temperature conditions. During the summer months, plants did not acclimate as well as at other times. The sequence of day/night temperature regimes which induced maximum acclimation was 20/15 C for 5 to 8 weeks; 15/5 C for 2 to 3 weeks; 15/5 C plus 1 hour of frost per day for 1 week. The duration of exposure to each temperature regime influenced the rate and intensity of frost-induced acclimation. Less than 5 weeks of warm temperature preconditioning at 20/15 C reduced subsequent frost-induced acclimation. The inductive influence of frost on cold acclimation was additive over 5 days of repeated exposure, but its effects after the first exposure(s) was immediate—requiring 1 to 4 days of 15/5 C following the frost treatments for the expression of the frost-induced acclimation to be manifest. There was a 75% increase in RNA following 3 days of frost exposure and plants in an O2-free atmosphere during frost exposure failed to acclimate. The results suggest that seasonal acclimation behavior was due to endogenous rhythms rather than developmental stage, and that the frost-induced phase of acclimation involves aerobic metabolic processes.

Cold acclimation has been induced artificially in plant species by a variety of treatments, usually involving a reduction of temperature and/or a shortening of photoperiod (1, 2, 4, 5, 7, 11, 14). Short photoperiods trigger the onset of the first of at least two stages of acclimation in several woody species (1, 4, 11, 14). Williams et al. (17) and McKenzie et al. (10) showed that this response is phytochrome-mediated in *Cornus stolonifera*. Temperatures during the short-day-induced phase of acclimation appear to be important (1, 14). During the first weeks of shortened photoperiods, warm temperatures (20/15 C day/night) are necessary for optimal hardening in *C. stolonifera* (1, 14). A burst of metabolic activity is observed during this phase of acclimation (7, 15), which is undoubtedly facilitated by the warm temperatures. After a period of short day and warm temperature preconditioning cold acclimation is continued by a period of cool nonfreezing temperatures (15/5 C day/night) (1).

Subfreezing temperatures (−3 to −5 C), which apparently trigger the second stage of acclimation, can in some plants induce full cold acclimation without such preconditioning (4, 5). In a number of hardy species, preconditioning through the first stage of acclimation (to hardiness levels of −18 to −25 C) enhances rapid and optimal acclimation (1, 2, 4, 12, 14) even though it may not be totally essential.

van Huystee (14) showed that *C. stolonifera* would not acclimate during the spring flush of growth. Evidence such as this led Levitt (7) to postulate that growth and development are inversely related to freezing tolerance. Schwarz (12) believed that seasonal rhythms, not developmental stage, are important in cold acclimation in pine. His studies led him to conclude that the internal rhythm of the plant, along with photoperiod and temperature, influence annual changes in freezing resistance. Howell and Weiser (4) observed several degrees of cold hardening in apple plants grown during the autumn in a warm greenhouse under long days. They suggested that endogenous rhythms or unmeasured environmental changes such as light quality might be responsible.

Siminovitch et al. (13) have also suggested the possibility that the hardiness and protoplasmic transformations which they observed in *Robinia pseudoacacia* were initiated by seasonal rhythms in the tree or environmental factors other than low temperature.

A myriad of metabolic changes has been observed throughout the course of acclimation (7, 15). The fact that many of these changes were observed during the first stage of acclimation led Weiser (15) to suggest that short day induction involves metabolic processes. In many studies, measurements of metabolic changes have been made over the entire course of acclimation with no attempt to determine whether changes occurred during the short day or the frost-induced stage, or both. There has been little research to determine whether frost induces metabolic changes in plants at the onset of the second stage of acclimation. These investigations were undertaken to examine that question, to study seasonal cold acclimation, and to develop a rapid method of artificially acclimating *C. stolonifera* plants at weekly intervals under a fixed photoperiod.

MATERIALS AND METHODS

A climatic race of *C. stolonifera* Michx. native to Dickinson, N.D. was propagated from a single clone by rooting stem tip cuttings. Rooted cuttings were transplanted into 15-cm pots in a soil:sand:peat (2:1:1) mixture and grown for 2 to 3 months in a warm (20/15 C, day/night) greenhouse at a 16-hr, long day photoperiod. The long day photoperiod was achieved by supplementing the natural daylength with mercury vapor lamps when necessary. During this growth period plants were pruned to two main leaders and trained vertically. At the beginning of the acclimation experiments plants of uniform size were transferred to controlled environmental chambers under short day conditions at a 9-hr photoperiod.

**Cold Acclimation.** Temperatures used to induce acclimation during the first stage of hardening were based on studies by

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Fuchigami et al. (1). The WT4 preconditioning phase consisted of a 20/15 C day/night regime. This was followed by a CT phase of 15/5 C. In order to induce the second stage of acclimation in the 15/5 C regime were exposed to 1 hr of frost (−5 C) during the dark period. This −5 C treatment was sufficient to freeze the plants. Frost-induced hardening at −5 C has been reported in several woody species (5, 11). The optimal length of each of these temperature regimes was studied and is reported herein.

**Hardiness Measurements.** Hardiness of stem tissues was evaluated by van Huystee's controlled freezing method (14). Sections (2.5 cm) of stem internodes were frozen at a rate of 6 to 8 C/hr. Test temperatures and freezing rates were monitored with a thermocouple inserted into the pith of one of four sections (one section taken from each of four plants) wrapped in aluminum foil, placed in a thermost flask, and frozen in a freezer set at −40 C. The temperature of the freezer was lowered slowly overnight at 5 C. Stem sections were then removed from the flasks and incubated in a humid environment at 20 C for 7 days before evaluation for injury. Viability was evaluated by visual observation of the browning of the stem tissues, particularly the cambium which was generally less hardy than the other tissues. Injury was rated on a scale of 1 (no damage) to 4 (severe damage).

Results of this method correlated well with whole plant and callus regrowth tests conducted in preliminary studies. A rating of 2.5 was found to correspond with the killing point of vital stem tissues. Hardiness levels shown in ensuing tables and figures are the lowest test temperatures at which the injury rating was <2.5.

Tender samples (hardy to −15 C or above) were inoculated with ice as described by McKenzie et al. (10) to avoid supercooling injury, and were removed at test temperatures at 3 C intervals. Stems hardy to below −15 C were frozen without ice inoculation and were removed at 5 C test intervals. Stems thought to be hardy to below −190 C were frozen to −80 C as described. They were then removed from the therms flask and submerged in liquid N2 for 5 to 10 min and allowed to equilibrate back to −80 C before returning them to the flask for slow thawing as described.

**Modified Atmosphere Treatment.** Studies in an O2-free atmosphere were accomplished by placing plants inside a transparent polyethylene bag (1.22 × .91 m). The bag was sealed except for an inlet and outlet valve through which gaseous N2 was allowed to flow at a rate of 100 ml/min. O2 level was monitored by passing the gaseous outflow through a small chamber equipped with an O2 electrode attached to a Beckman model 777 O2 analyzer. Plants were then placed in the bags inside a controlled environment chamber and subjected to a −5 C frost for 1 hr after the O2 content had reached its minimum level (0.1%). They were left under anaerobic conditions for a total of 3 days, during which time they received three successive frost treatments at 24-hr intervals. The plants were then returned to normal atmosphere for 4 days at 15/5 C, after which time the hardiness was evaluated.

**Nucleic Acid Analysis.** Nucleic acids were extracted from bark tissues according to the scheme outlined by Gusta and Weiser (3). Since nucleic acid fractionation was accomplished by gel electrophoresis rather than MAK chromatography, smaller amounts of lyophilized, ground tissue (0.25 g) were required and the volumes of extraction solutions were reduced by one-half (3). The nucleic acid precipitate was dissolved in 0.15 M sodium acetate (pH 6) containing 0.5% sodium lauryl sulfate, and reprecipitated with 2 volumes of ethanol. After centrifugation, the pellet was washed with cold 80% ethanol and dissolved in 0.5 ml of electrophoresis buffer supplemented with 0.2% highly purified sodium lauryl sulfate and 5% sucrose. Acrylamide gels (2.4%) prepared as described by Loening and Ingle (9) were layered with 50 μl of the RNA solution and allowed to run 1.5 hr. After running, gels were scanned at 260 nm on a Beckman DU spectrophotometer equipped with Gilford linear transport to resolve RNA peaks. The spectra were recorded and total RNA was reported in terms of total peak area in cm2.

**RESULTS AND DISCUSSION**

**Cold Acclimation.** Figure 1 shows the optimal sequence of temperature regimes for artificially inducing cold acclimation in C. stolonifera plants at a 9-hr photoperiod. The levels of hardiness achieved over the course of such a sequence of treatments are shown in Figure 2. There were variations in the levels of hardiness achieved in different runs during different seasons and with different timings and durations of temperature regimes. We found hardiness levels of −10 to −12 after the WT preconditioning regime, −17 to −22 after the CT regime, and −40 to −50 after the frost treatment.

**Photoperiod.** A 9-hr photoperiod was used as a short day treatment because preliminary studies showed that longer photoperiods were only marginally successful in inducing the first stage of acclimation. Results were similar in previous studies (1, 10, 14). A photoperiod of 9 hr is apparently short enough to induce acclimation in the North Dakota clone of C. stolonifera, and yet long enough to provide adequate radiant energy for the metabolic processes which occur during acclimation.

**WT Preconditioning.** The optimal length of the WT preconditioning regime was found to be 5 to 6 weeks (Table I). Less than 5 weeks failed to prepare plants sufficiently to respond optimally to subsequent frost induction. For example, 4 weeks of WT preconditioning ultimately resulted in hardiness levels of only −16 C, while 5 or 6 weeks of WT preconditioning produced levels of −41 C. More than 6 weeks of WT preconditioning appeared to be

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**Fig. 1.** Optimal sequence of temperature regimes for artificially inducing cold acclimation in C. stolonifera plants at a 9-hr photoperiod.

**Fig. 2.** Hardiness levels of stems of C. stolonifera plants artificially acclimated at 9-hr photoperiod for 5 weeks at 20/15 C (WT), followed by 3 weeks at 15/5 C (CT) followed by 1 week of CT with a 1-hr nightly frost.
superfluous; the rate of acclimation decreased and tended to level off beyond the 6th week (Fig. 3).

**CT Induction.** Figure 3 shows the influence of CT treatment on stem hardness following the WT preconditioning. Plants placed in CT after 5 weeks of WT treatment maintained a steady rate of acclimation (Fig. 3b), while the acclimation rate of plants which remained in WT decreased and began leveling off (Fig. 3a). These results confirm an earlier report (4) that short days and WT alone cannot induce full hardness, but that CT above freezing can eventually induce substantial hardness. Dogwood plants (Table I) which achieved at least –17°C of hardness in the first stage were capable of hardening to –40°C with subsequent frost exposure. Less hardy plants (–14°C) responded little to the frost treatment. Our studies showed that the length of CT treatment required to achieve a hardness level of –17°C was generally 2 to 3 weeks. Glerum (2) observed that hardening in conifers occurred in two stages, and that the line of demarcation occurred at about –18°C in nature.

**Frost Induction.** Comparison of hardness plots (b) and (c) in Figure 3 illustrates the added effect of frost (–5°C) on hardness induction in plants which have been exposed to 3 weeks of CT treatment following the WT preconditioning. One week of CT with exposure to a nightly frost of 1-hr duration increased the hardness level from –17°C to –40°C, while 5 additional weeks of CT without the frost treatment were required to induce hardness to –53°C. Experiments to determine the influence of repeated frost on cold acclimation showed that one frost treatment induced about an additional 10°C of hardness (Fig. 4). Additional frost treatments of 2, 3, and 5 days increased hardness linearly to –70°C provided the treatment groups were retained at 15/5°C for the remainder of a 7-day experimental period following their respective frost treatments. Plant groups which received no follow-up of 15/5°C, but were sacrificed and evaluated for hardness immediately following their respective frost treatments of 2, 3, or 5 days duration showed a leveling off of induced hardness after the 2nd and 3rd days of frost exposure (Fig. 4). The frost induction of hardness was not immediate but required time at 15/5°C for optimum expression.

**Seasonal Effects.** In the course of these investigations frost treatments failed to promote hardness in several studies. These failures invariably occurred during the summer months, June through August. This is illustrated by the plot in Figure 5. By contrast, photoperiodic (short day) induction was not influenced by seasonal factors as shown by the X plot in Figure 5. McKenzie
et al. (10) found that plants transferred to growth chambers during the period from August to December required 2 to 6 weeks longer to acclimate fully than at other times of the year. This might have occurred because he used a short (4-week) WT preconditioning treatment. The O plot of natural acclimation in Figure 5 shows that the hardiness of plants in the field was -3 C at the time of the year when frost failed to induce hardness under controlled (growth chamber) conditions. Just as field plants began to increase in hardiness in the autumn months, so plants under artificial acclimation began then to respond to frost induction. The timing of natural deacclimation in the spring was also correlated rather closely with the failure of frost to induce hardiness in growth chamber-hardened plants (Fig. 4).

The fact that there appears to be a seasonal rhythmic pattern in the acclimation of dogwood is not surprising. Schwarz (12) stated that seasonal rhythms rather than developmental stage control hardiness, and Siminovich et al. (13) suggested that seasonal rhythms rather than low temperature per se are controlling factors in hardiness. In dogwood, Howell et al. (4) and McKenzie et al. (10) suggested that endogenous rhythms might be involved in observed acclimation patterns.

Unexpectedly, the seasonal influence was on the frost-promoted stage of acclimation rather than on the photoperiodically induced stage of acclimation (Fig. 5). This implies that the seasonal influence on acclimation may indeed be due to endogenous factors. Lapin (6) stated that there is a strict seasonal regulation of these rhythms controlled by an internal system of regulation. In reference to seed dormancy Went and Sheps (16) stated that although temperature and light affect dormancy, they are often interrelated to an "endogenous clock." Cold acclimation and dormancy are vital processes in plant survival, and both are affected by the same environmental factors. It is possible that the endogenous factors influencing these processes may also be similar. The plants used in this study were grown under regulated greenhouse conditions. We should not discount the possibility that external factors not monitored in this study such as CO2, O2, or O3 levels, plant temperatures, radiant energy, or light quality, may change with the seasons and influence acclimation.

Nature of Frost Induction. Because of the physical nature of frost, it is tempting to conclude that second-stage acclimation is a physical rather than a metabolic process (15). Results in Figure 4 suggest that it might be both. Plants became hardy to -33 C after one frost exposure whether hardness was measured immediately following frost or after 6 additional days of incubation at 15/5 C. The rapid increase in hardness after one frost did not seem to involve metabolic processes since the response was so rapid, and additional time (6 days) did not enhance acclimation. Metabolic involvement did seem likely in the case of additional frost treatments. There was no immediate increase in hardness following frost treatment on the 2nd, 3rd, or 5th days, but those frosts did substantially increase hardness when plants were incubated for the remainder of the week at 15/5 C (Fig. 4), a period of relatively warm temperatures during which metabolic activity might occur.

Krasavtsev (5), using subzero temperatures for hardening, stated that "slow biochemical and structural changes in the protoplasm take place in fruit trees subjected to weak frosts." Weiser (15) suggested that the second stage of acclimation may be "metabolic, physical, or both." Results of this study lend credence to the latter.

Plants exposed to frosts for 3 nights under anaerobic conditions (N2 atm) failed to acclimate beyond the level of the nonfrosted controls (-18 C) (Fig. 6). However, plants given the same inductive frosts under aerobic conditions acclimated to -30 C. A control experiment in which tender plants were placed under similar anaerobic conditions, but with no frost induction, showed no loss of viability as a result of the 3-day N2 treatment. These results suggest that aerobic metabolism is directly involved in the acclimation process.

In studies on nine woody species, Rakitina (11) concluded that aerobic conditions were necessary for acclimation at subzero temperatures. He also showed that disruption of normal gas exchange not only impaired the capacity of plants to harden but caused a loss of hardness already achieved.

Ribosomal RNA in C. stolonifera bark increased during the first stage of acclimation under field conditions between early September and mid-October when stools hardened from -10 to -19 C (Fig. 7). By late November plants in the field became hardy to -70 C. Similar rRNA increases have been observed in apple (8) during cold acclimation. In mid-October plants from the field (hardy to -19 C) were placed in a growth chamber and given three frost treatments on successive days. As shown in Figure 7, this frost treatment caused a dramatic increase in rRNA.

These studies illustrate that the frost-induced stage of acclimation in dogwood is influenced by several factors, the most basic of which are photoperiod and temperature. Seasonal rhythms influ-
ence this stage of hardening which apparently depends in part on active metabolic processes.

**LITERATURE CITED**

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