Induction of Cold Acclimation in *Cornus stolonifera* Michx.[1,2]

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ABSTRACT

A warm (20 to 15 Celsius day or night) preconditioning treatment enhanced cold acclimation of *Cornus stolonifera* bark under short-day conditions when plants were preconditioned for at least 4 weeks. Warm preconditioning inhibited the acclimation of plants subjected to long photoperiods. Removing leaves from plants exposed to low temperatures and short days inhibited acclimation. Removal of buds did not affect acclimation. Plants did not acclimate unless they were exposed to at least 4 weeks of short photoperiods prior to defoliation. Plants began to acclimate to cold at the time of growth cessation but not before. When half of the leaves were removed from plants, the defoliated and foliated branches both acclimated as well as branches on completely foliated plants. Girdling the phloem between foliated and defoliated branches prevented acclimation of the latter regardless of the position of the girdle in relation to the root system and the defoliated branch. When all of the leaves of plants were covered with aluminum foil to exclude light after 0 or 4 weeks of exposure to short days, the results resembled a defoliation study, *i.e.*, plants with leaves covered at the start of the experiment failed to acclimate, and those covered after 4 weeks acclimated to some extent but less than uncovered control plants. Under long-day conditions plants with all leaves covered failed to acclimate, and plants with none or half of their leaves covered acclimated equally and to a limited extent. Under short-day conditions, however, the covered branches of partially covered plants acclimated more than their uncovered counterparts or branches of totally uncovered plants.

In nature the living bark of many woody plant species acclimates to cold in two stages during the autumn (5, 7). The results of controlled environment studies have indicated that the first stage of acclimation is induced by short days (7, 8, 18) and the second stage by low temperatures (5, 18). Russian workers have hypothesized that there is a third stage or type of acclimation which is induced by prolonged exposure to very low temperatures in midwinter (15).

Results of controlled environment studies have also suggested that endogenous rhythms influence cold acclimation. During the spring growth-flush, *Cornus stolonifera* plants cannot be induced to acclimate regardless of environmental conditions (3, 18). Conversely, apple trees grown in a warm greenhouse under artificially long photoperiods in the autumn acclimate to some extent in spite of the supposedly noninductive environment (5).

Recent studies of the first stage of acclimation in several deciduous woody species have revealed that leaves exposed to short days are the source of a translocatable hardiness-promoting factor(s) (5, 8), and that leaves exposed to long days are a source of factors which inhibit hardiness (5, 8). Removal of leaves from short-day induced plants inhibits bark acclimation, whereas removal of leaves exposed to long days has the opposite effect (5, 8). Grafting studies with diverse climatic races of *C. stolonifera* have demonstrated that the hardiness-promoting factor(s) from the leaves of one genotype can enhance cold acclimation in the bark tissues of another genotype (3). Irving and Lanphear (8) have reported that the hardiness-promoting factor(s) is translocated from leaves to overwintering tissues via the xylem.

Studies in which different parts of the same plant are exposed to different environments have shown that the low temperature-induced phase of cold acclimation does not involve a translocated factor(s) (5). In spite of this basic difference, either low temperature or short days can induce acclimation in the absence of the other inductive factor. Plants exposed to low temperatures and long days will, in time, acclimate fully. Similarly, exposure of plants to short days and relatively high temperatures results in substantial, although not full, hardiness (18).

Even though these studies have contributed considerably to our knowledge of the first stage of acclimation, there are still a number of unresolved questions. For example, the influence of leaf factors on bark acclimation has been characterized, but little is known about the possible influence of root, bud, or stem factors. Buds have been shown to be the photosensitive perceptor sites which are involved in the breaking of dormancy in *Fagus sylvatica* L. (10), and leaves or buds are known to be the site of perception of light for the onset of physiological rest in many plant species (2, 9, 19, 20). The translocation path of the hardiness-promoting factor(s) from short-day induced leaves to the overwintering tissues has not been studied thoroughly. The roles of temperature and photoperiod have been studied during the acclimation process (6–8, 18), but we know little about how the developmental status or the previous environmental history of the plant may affect its ability to acclimate.

This study was designed to examine the possible role of bud, root, and stem factors in cold acclimation and the translocation route of hardiness-promoting factor(s) and to assess the influence of environmental preconditioning on the first stage of cold acclimation.

MATERIALS AND METHODS

Two clonally propagated northern races of *C. stolonifera* Michx. native to Minnesota and North Dakota were used in the 12 studies (13). All plants in each experiment were of a single

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clone. Prior to study plants were actively growing in a warm (24 C) greenhouse at a 16-hr photoperiod. In growth chamber studies, the light was supplied by a mixture of cool white fluorescent and incandescent bulbs. The intensity at pot height was 8.2×10^4 ergs/cm² sec at 15 C as measured by a YSI Kettering model 65 radiometer. Short-day treatments consisted of natural autumn photoperiods in field studies and either 10- or 12-hr photoperiods in the growth chamber studies. Either the 10- or 12-hr photoperiod causes growth cessation and induction of rest period in both of the clones studied. The long-day treatments in both field and growth chambers consisted of the short photoperiod plus a 3- to 4-hr interruption of the middle of the dark period with light from incandescent bulbs.

Unless otherwise indicated, day-night temperature regimes in growth chamber studies consisted of a 20 to 15 C preconditioning period followed by 15 to 5 C for inducing acclimation. All of the treatments and hardiness tests were run in triplicate. To test hardiness, stems (internodes) were cut into 2-cm-long internode segments and were subjected to a controlled freezing test as previously described (3). Internode segments were frozen in Dewar flasks at a rate of 10 C per hour. Flasks were removed from the freezer at 2-C intervals; the internode segments were rewarmed and incubated in a humid chamber at room temperature. After 7 days the samples were scored for survival. Any segments not showing discoloration and breakdown of cells in the cambium or bark were rated as living. Experience has shown that living samples are capable of forming callus when incubated further for 20 to 30 days. Freezing tests were run in triplicate, and in these studies there was almost always perfect agreement in the results of survival scoring on all three segments from each treatment at each test temperature. In rare instances when all samples did not agree, the atypical sample was disregarded. Hardiness is expressed in figures as the lowest temperature at which samples were uninjured (lowest survival temperature). Growth was evaluated by periodically measuring stem length and by averaging the measurements. Differences in final growth between treatment means were evaluated by Duncan's multiple range test (14).

RESULTS

For clarity the 12 studies conducted are referred to by number in the ensuing discussion. In the interest of brevity, data are presented in Figures for only six of the 12 studies conducted, and the results of the other six supporting studies are discussed briefly.

Study 1 indicated that low preconditioning temperatures not only failed to enhance acclimation but, in fact, inhibited the process. Three plants were subjected to short days and a low day-night temperature regime of 15 to 5 C continuously for 40 days. Another lot of three plants was subjected to higher day-night temperatures (20 to 15 C) for 28 days before they were transferred to 15 to 5 C for the balance (12 days) of the 40-day experimental period. Plants given the warm preconditioning treatment grew more but also became more cold resistant (-16 C) than those exposed continuously to low temperatures for 40 days (-10 C) (Fig. 1).

In study 2 the influence of the duration of a warm preconditioning treatment (20 to 15 C) was examined. Ten lots of three plants each were subjected to either 0, 1, 2, 3, or 4 weeks of preconditioning at either long or short photoperiods before they were transferred to short days and a 4-C constant temperature for an additional 17, 16, 15, 14, or 13 weeks, respectively. The hardiness of plants in the 10 treatments was compared at the end of 17 weeks. Figure 2 shows the results.

As in the previous study, a warm preconditioning period (20 to 15 C) for 4 weeks resulted in effective acclimation (to -26 C) under short-day conditions. When there was less than 4 weeks of preconditioning, acclimation was not enhanced; in fact, no pre-
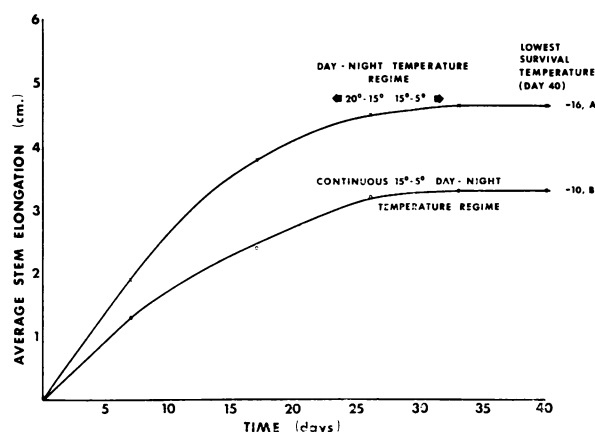

FIG. 1. The influence of preconditioning temperatures on the elongation and hardiness of *C. stolonifera* stems. The hardiness (lowest survival temperature) was determined on the 40th day of the experiment. The letters A and B indicate that the average stem elongation was significantly different at the 0.05 level of significance as determined by the Duncan multiple range test. Temperature regimes were as shown.

conditioning at all was better than 1 or 2 weeks. Under long-day conditions plants did not become as hardy, and plants acclimated less as the duration of preconditioning was increased.

The influence of a higher preconditioning temperature regime (30 to 25 C) was examined in a parallel companion trial (study 3; data are not shown). In all treatments the hardiness was within 2 C of that observed in the comparable treatments of study 2, and the relationship of hardiness to the duration of preconditioning was also the same.

In study 4 buds and leaves were manually removed from 60 plants in the field on August 8, 1968. Any regrowth of buds or leaves was removed throughout the course of the study. The hardiness of unaltered plants and plants with leaves and buds removed was evaluated at five dates during the autumn (Fig. 3). Three plants were sampled in each treatment at each sampling date. Manually defoliated plants were less hardy than their foliated counterparts at all sampling dates, and they were dead by the final sampling date on November 14. The presence or absence of buds did not affect the hardiness at any of the sampling dates.

A parallel companion study (study 5, data are not shown) was conducted to examine the influence of bud and leaf removal on acclimation under long-day conditions. The experimental design was the same except plants were exposed to 3 hr of light in the middle of the night. The exposure to long-day treatment in this study resulted in about 6 to 8 C less hardiness in foliated plants at all sampling dates. Defoliated plants failed to acclimate (killed below -4 C), and they were dead at the time of the final sampling on November 14.

In study 6 leaves were manually removed from 99 plants in a growth chamber at weekly intervals for 6 weeks and at 2-week intervals thereafter through the 12th week. All plants were exposed to short days and a day-night temperature regime of 20 to 15 C for the first 4 weeks and 15 to 5 C thereafter for 10 additional weeks. Three plants were sampled in each treatment at each sampling date. Hardiness tests and stem length measurements were made at weekly intervals for 14 weeks (Fig. 4).

There was no cold acclimation before growth cessation. Leaf removal during the first 4 weeks of the study resulted in reduced stem elongation, and plants in those treatments failed to acclimate (killed below -4 C). Stems of plants in these treatments began dying back from the tips after the 12th week. Plants stopped growing at about the 5th week, and those that were defoliated anytime after that time acclimated. Acclimation was greater as the time of defoliation was delayed. Those defoliated at the 5th

ENVIRONMENTAL TREATMENTS				LOWEST SURVIVAL TEMPERATURE AFTER 17 WEEKS (°C)
PRECONDITIONING TREATMENT			LOW TEMPERATURE (4°C)	
Daylength	Temperature, °C (D−N)	Duration (weeks)	SHORT DAYS (10 hours) (Duration weeks)	

FIG. 2. The influence of the duration of warm (20 to 15 C) preconditioning treatments at LD and SD on subsequent cold acclimation of plants subjected to short days and 24 C. Hardiness (lowest survival temperature) was measured after 17 weeks.
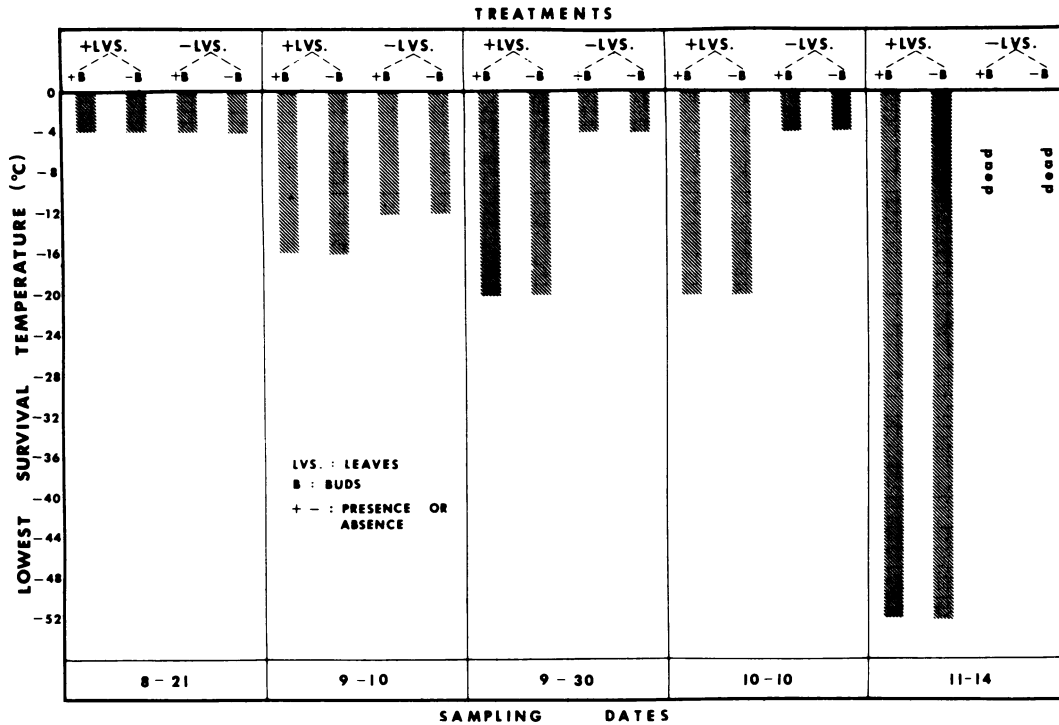

FIG. 3. The influence of leaf and bud removal on the cold acclimation of *C. stolonifera* in the field. Leaves and buds were removed manually as indicated on August 8. Sampling dates indicate times at which hardiness determinations were made during the fall of 1968. "Dead" indicates that defoliated plants had died in the field before the November 14 sampling date.

week became hardy to −16 C, whereas those defoliated at the 12th week acclimated to −30 C.

In several other defoliation experiments, collectively referred to as study 7 (data are not shown), plants in a growth chamber which provided long days and a 20 to 15-C temperature regime were enclosed in polyethylene bags and exposed to ethylene gas over a range of concentrations from 50 to 1000 μl/liter for 12 hr once every 3 days. Treatments at the higher ethylene concentrations promoted senescence and abscission of older leaves to some extent. Notes were made of ethylene-induced leaf abscission every 3 days, and comparable leaves were manually removed at each date from plants that had not been treated with ethylene. After more than half of the leaves had abscised in several of the treatments the growth chamber was reprogrammed to provide short days and a temperature regime of 15 to 5 C. Periodic hardiness tests of plants defoliated manually or by ethylene treatment revealed no differences.

In study 8 leaves were manually removed from single branches or whole plants in the field on July 30, as shown in Figure 5 A. In some treatments, branches were girdled by removing a 1.5-cm

band of bark tissue and scraping the exposed woody surface with a sharp scalpel. There were three plants in each treatment. The hardiness of plants and branches in the various treatments was evaluated on October 10.

Foliated plants and branches acclimated to −16 C. Completely defoliated plants failed to acclimate (−4 C). Defoliated branches on partially defoliated plants acclimated to −16 C if they were ungirdled and they failed to acclimate if the phloem was girdled between the defoliated and foliated parts of the plant (Fig. 5 A).

Figure 5 B shows the results of a similar growth chamber trial (study 9) in which plants were girdled and partially defoliated before exposure to short days and a day-night temperature regime of 15 to 5 C. Hardiness was tested after 37 days. As in the previous study defoliated branches that were girdled failed to acclimate. This was true whether the root system was isolated by girdling from the defoliated branch or not.

In study 10 the leaves of plants or branches were covered with aluminum foil to exclude light as shown in Figure 6. Plants were exposed to short days and to the standard temperature regimes (20 to 15 C for 4 weeks followed by 15 to 5 C for 8 weeks). Leaves
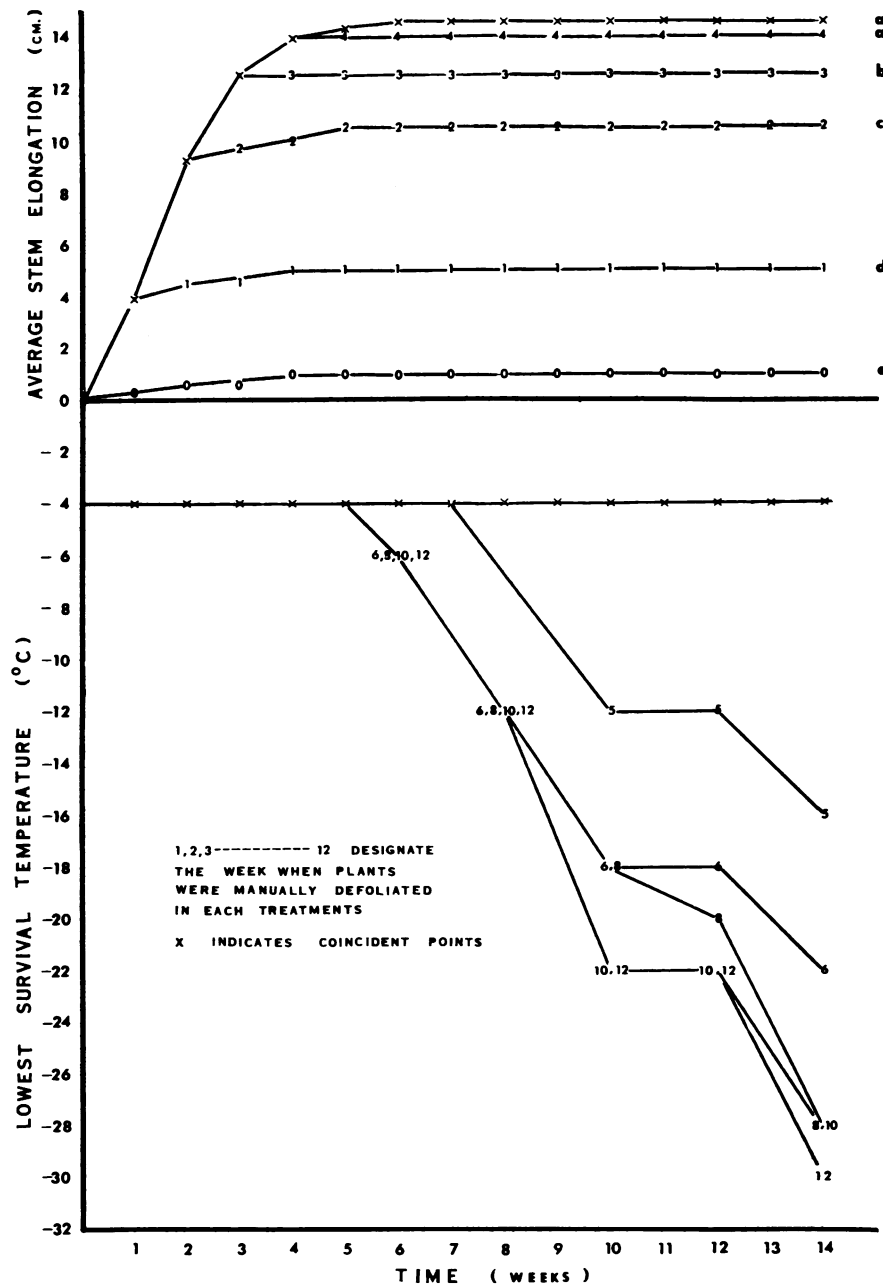
FIG. 4. The influence of weekly leaf removal on stem elongation and cold acclimation of *C. stolonifera*. Numbers (0–12) with each line indicate the number of weeks after the start of the experiment at which leaves were removed. Time refers to the weeks after the start of the experiment when growth and hardiness determinations were made. Letters (a to e) in common indicate that differences were not significant at the 0.05 level by the Duncan multiple range test.

were covered individually at either the beginning of the study or after the first 4 weeks. Hardiness determinations were made at the 4th, 8th, and 12th week of the study.

When leaves were not covered, the plants acclimated to −8 C by the 8th week and to −28 C by the 12th week. Plants with all the leaves covered at the beginning of the study did not acclimate (killed below −4 C). Plants with all the leaves covered after 4 weeks acclimated to a limited extent. They were hardy to −8 C by the 8th week and to −10 C by the 12th week. Covered leaves senesced and abscised sooner than uncovered leaves. These results are essentially what would be expected if leaves had been removed instead of covered. However, when the leaves on one branch of a plant were covered and those on the other branch were not, the results were not similar to those caused by defolia-

tion. Branches with covered leaves became hardier than branches in the other treatments. By the 8th week both branches of partially covered plants were hardy to −14 C. This was six degrees hardier than branches of plants with no leaves covered. At the 12th week the branches with covered leaves on partially covered plants were not killed at the lowest test temperature (−32 C). Branches on the same plants without covered leaves were less hardy (−16 and −20 C). The branches of plants on which no leaves were covered were hardy to −28 C.

Two additional leaf covering studies (studies 11 and 12) were conducted (data are not shown). Study 11 was the same as study 10 except the leaves were covered only after 4 weeks. The results were similar to those of study 10. After 12 weeks the plants with either uncovered or covered leaves were hardy to −32 and −20 C,
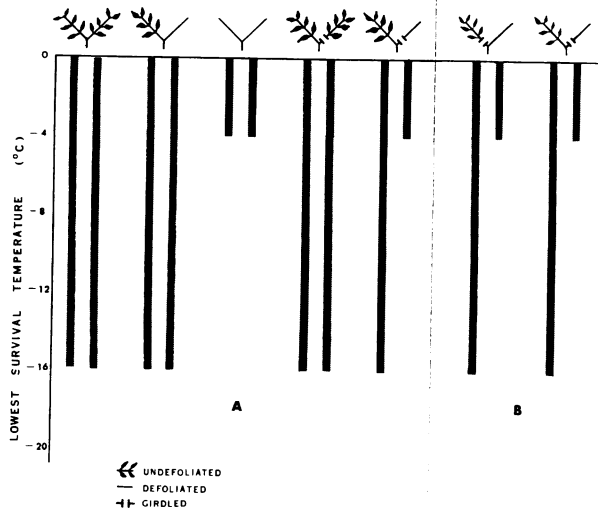
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FIG. 5. The influence of girdling and leaf removal on the cold acclimation of *C. stolonifera*. A: Experiment conducted in the field. Treatments were given July 30, and hardiness was tested October 10. B: Experiment conducted in a growth chamber under short-day (8-hr photoperiod), and low temperature (15 C day and 5 C night) conditions. Hardiness was determined after 37 days. The illustrations on the abscissa depict the treatments and parts of plants tested for hardiness.

respectively. The covered leaves of branches from partially covered plants were hardy beyond −40 C, whereas the uncovered side was hardy to only −22 C.

DISCUSSION

Natural cold acclimation in *C. stolonifera* appears to be a sequential process which proceeds most effectively when each inductive phase is completed before proceeding to the next. Studies 1, 2, and 3 indicate that exposure to relatively high temperatures (20 to 15 C or 30 to 25 C) during the early phases of short-day induction enhances acclimation. Although it has been reported previously that simultaneous exposure of plants to short days and low temperatures does not always result in optimal acclimation (18), the cold hardiness-promoting influence of high temperatures is not generally recognized. These results support the view that the early stages of acclimation are dependent upon active metabolic processes. This viewpoint is also circumstantially supported by the observation of many metabolic changes which occur in the bark of *C. stolonifera* during this period (11, 12, 16–18). In a recent (unpublished) study we have also found that inhibitors of oxidative phosphorylation and nucleic acid synthesis (transcription) interfere with short-day induced growth cessation in *C. stolonifera* explants, *i.e.*, the inhibitors promote growth at short photoperiods.

Studies 2 and 3 also indicate that the duration of the warm preconditioning treatment is critical, and the results of study 6 suggest that plants (leaves) must receive a certain number of short

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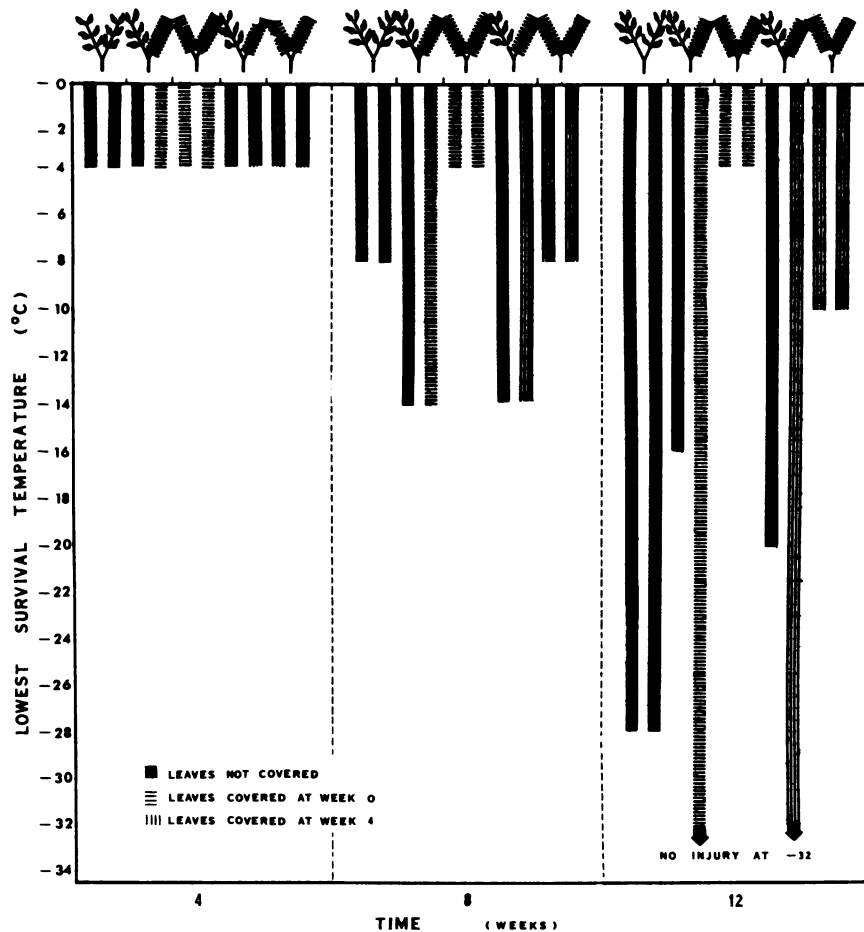IIII LEAVES COVERED AT WEEK 4

NO INJURY AT −32

FIG. 6. The influence of leaf covering on the controlled cold acclimation of *C. stolonifera*. Individual leaves on the branches indicated were covered with aluminum foil. Illustrations on the abscissa show treatments and part of plant tested for hardiness. Time refers to the weeks from the start of the experiment at which hardiness tests were run.

days for acclimation. It has been proposed that many of the photoperiod- and temperature-controlled responses of plants are related to the re-establishment of endogenous rhythmic patterns (4). Time is required for plants which are conditioned to a particular day-night periodicity to establish new response patterns when the environment changes. Until new patterns are well established, physiological responses may be inhibited or delayed (4). This may explain why hardiness is inhibited in plants exposed to new temperature (studies 2 and 3) or photoperiod regimes for insufficient periods of time.

Growth cessation is a prerequisite to cold acclimation (study 6), and the induction of growth cessation is probably one of the prime functions of short days in the natural cold acclimation of plants. Conditions which promote growth, such as the warm preconditioning treatments combined with long photoperiods in studies 2 and 3, invariably inhibit acclimation. The nature of the obligatory relationship between growth cessation and acclimation which exists in woody but not herbaceous (1) plants is probably complex. One simple possibility which cannot be ignored is that growing plants may become depleted in the energy-producing substrates required for acclimation. This possibility is not unlikely under warm, long-day conditions in poorly illuminated growth chambers. Removal of leaves from plants in warm environments would also deplete reserves, and the poor acclimation and die-back or death of defoliated plants at the later sampling dates in studies 4, 5, and 6 may be attributable to this situation. Removal of leaves from several woody species under long-day conditions has been reported to enhance acclimation (by removing the source of hardiness inhibiting factors) (5, 8). This effect was not observed in C. stolonifera (studies 5 and 7). It is difficult to judge whether C. stolonifera is different from the species previously tested, whether the previous results are in error, or whether the effect was masked because defoliated plants became depleted in reserves.

Although short-day induced leaves were necessary for efficient acclimation, studies 4 and 5 indicated that buds are not involved, and studies 8 and 9 discounted the possibility of phloem-transported root factors in acclimation. Results of study 7 indicate that ethylene-induced leaf senescence and abscission have the same inhibitory effect on acclimation as manual leaf removal.

The results of the leaf removal studies (studies 4, 5, 6, 7, 8, and 9) support the concept that the short-day leaf is the source of a hardiness promoting factor(s) (3, 5, 8), and the girdling experiments (studies 8 and 9) clearly indicate that this promoting factor(s) is translocated in the phloem. This finding is contrary to a previous report by Irving and Lanphear that transport is in the xylem (8). However, the previous suggestion was based on observations that a girdled branch exposed to long days acclimated almost as well as a branch on the same plant exposed to short days when both branches received the same prolonged exposure to low temperature (4 weeks at 4.4 C + 1 week at −1.1 C + 1 day at −2.2, −3.3, −4.4, and −5.5 C). In view of the long, low temperature treatment, the acclimation of the girdled, long-day branch should not have been unexpected because these same

workers (7), and others as well (5), have shown that low temperatures can fully acclimate woody plants exposed to long days.

The unusually effective acclimation of branches with aluminum foil-covered leaves on plants where only some of the leaves were covered (studies 10 and 11) is as interesting as it is puzzling. Since the foil covering caused leaves to senesce, it is possible that some substance produced by senescing leaves enhances acclimation. If this is true the substance must be translocated to a limited extent only inasmuch as its hardiness-promoting effects were apparent only on the covered side of the plant. The results of study 7 indicate that this hypothetical "senescence factor" is not ethylene or a by-product of ethylene-induced senescence, and a comparison of study 12 with studies 10 and 11 indicates that short-day factors are also involved in this unusually effective acclimation response.

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