Plant Physiol. (1967) 42, 1191-1196

Environmental Control of Cold Hardiness in Woody Plants[1]

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Received April 17, 1967.

Summary. The development of cold hardiness in 2 woody plant species (*Acer negundo* and *Viburnum plicatum tomentosum*) was shown to be independent of the induction of bud dormancy. Substantial hardiness levels were obtained under controlled conditions with long days and certain low temperatures—without dormancy development as a prerequisite.

Low temperatures given during the dark period with long days induced hardiness to a level not significantly different from that of short days. Giving plants continuous 10° temperatures under long days forced plants to harden as if they were under short days, even though they were not dormant.

Development of hardiness was shown to be a photoperiodic response. Increasing weeks of short days, followed by a low temperature hardening period in darkness, brought about a progressive increase in hardiness. The short day stimulus could be reversed by long days. Following 6 weeks of short days, the rate of hardening in darkness at 5° was over twice that of plants previously exposed to long days.

The chronological similarity between bud dormancy or rest (i.e. the inability to produce normal growth even under favorable growing conditions) and cold hardiness development in woody plants led observers to believe that these 2 processes were intimately associated (3, 9). According to Chandler (1), development of cold resistance was partly due to early or rapid development of the dormant period in the woody plant during late summer and early fall. He postulated, therefore, that substances move into the bark of the trees during the fall period as precursors of substances inducing cold hardiness, but that these materials could not accumulate until the plant was fully dormant. Thus, the theory evolved that cold hardiness could be induced only after the dormant state had been reached. This dormancy prerequisite concept indicated that the final state was the result of a 2-step process, first dormancy induction and then cold hardiness development. The idea gained acceptance even in the face of other research indicating, at least indirectly, that bud dormancy was not involved (2, 4, 7). This paper demonstrates that cold hardiness development is not dependent on bud dormancy.

Materials and Methods

Plant Material and Experimental Conditions. The plants used in this study included rooted cuttings of *Viburnum plicatum tomentosum* Thunb. (Doublefile viburnum) and seedlings of *Acer negundo* L. (Box elder). Plants were grown for at least 3 months at approximately 21° in a greenhouse under long photoperiods before undergoing experimental conditions.

Plants exposed to long photoperiods under fall conditions received 400 ft-c of artificial light from 5:00 PM until 11:00 PM. All other experiments were conducted under controlled environments at either 2500 or 1000 ft-c, the latter being used when 5° temperatures were employed.

Experimental Design and Analysis. A completely randomized design was used and the analysis of variance was performed, according to the procedure of Le Clerg et al. (6), on individual killing points to determine which variables were significant. Duncan's new multiple range test was utilized for mean separation. Those killing points followed by an identical letter are not significantly different at the 0.05 probability level.

Artificial Freezing Test. A standard freezing procedure was used in hardiness determinations. An increase in hardiness was represented by the ability of the tissue to survive a lower temperature. At least 8 tissue samples from each treatment were used. A 16 cm section from each plant was cut into 6 equal pieces and each was exposed to different temperatures. One section at 5° served as the control. The others were placed in styrofoam boxes in a freezer at −6.5°. Internal air temperatures were recorded in each box at 2 and one-half minute intervals. When the box temperatures reached −5° all the boxes, except one, were transferred to a freezer set at −12.5°. This process was repeated at −17.8°, −23.5°, and −29.0°.

[1] Journal Paper 3055, Agricultural Experiment Station, Purdue University, Lafayette, Indiana. Part of a Ph.D. Thesis, submitted by R. M. Irving.

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The rate of temperature drop was 3° per hour. After 2 hours at each temperature, the boxes were removed, held at 5° and the material was allowed to thaw. The samples were then placed in a plastic container under high humidity at room temperature for 36 hours.

Triphenyl Tetrazolium Chloride Technique for Viability Determinations. The viability of the frozen tissue and the extrapolation of the killing points were determined by using a slight modification of the triphenyl tetrazolium chloride (TTC) technique as outlined by Steponkus (8).

Previously frozen plant material was weighed to 50 mg samples. The *Viburnum* sections were cut to a maximum of 5 mm, while the *Acer* sections were cut to a maximum of 2 mm to facilitate penetration of the TTC into this species. (The remainder of each section was placed in a box of vermiculite which was then placed under an intermittent mist system. Visual determinations of viability was made after 10 days as a final check of the refined TTC test.) Tissue samples were placed in test tubes and 3.0 ml of a 0.6 % TTC solution (buffered at pH 7.4 in 0.05 M phosphate-phosphate buffer, plus 0.01 % Ortho *X-77* as a wetting agent). *Viburnum* samples were vacuum infiltrated at 12 cm of Hg for 2 minutes while *Acer* samples were vacuumed at 5 cm of Hg for 5 minutes.

Tubes were then stoppered and incubated at 27° for 15 hours.

The TTC solution was removed and the tissue rinsed with distilled water to remove any TTC not fixed in the sample itself. *Viburnum* samples were then diced to 2 mm sections to allow for a more quantitative extraction.

Tubes were filled to 7 ml with 95 % ethanol and placed for 10 minutes in a boiling water bath to extract the reduced TTC.

Tubes were cooled and filled to 10 ml with ethanol.

Absorbance at 530 mμ was recorded and the values divided by the absorbance of the 5° control to determine the percent reduction. The killing point was extrapolated as the temperature at which 50 % of the tetrazolium reducing capacity was lost. The value of 50 % was determined as the critical level in preliminary correlation studies between tetrazolium activity and visual observation of tissue viability.

Determination of the Dormant Condition. Plants were considered dormant if normal leaves were not produced within 5 weeks after transfer to favorable growing conditions. If a majority of the plants within a particular treatment failed to grow, that treatment was considered dormant.

Results and Discussion

Hardiness Development Under Natural and Long Day Conditions. To clarify the role of dormancy in cold hardiness, an experiment using *Viburnum plicatum tomentosum* as the test plant was designed that would indicate the killing point (KP) of the non-dormant, non-hardened; dormant, non-hardened; non-dormant, hardened; and dormant, hardened plants. The treatments and the results obtained are shown in table I. Plants located in the greenhouse from September 15 to December 15 under long days (LD) (non-dormant, non-hardened) were killed at −6.0°. Plants located in the greenhouse under short days (SD) (dormant, non-hardened) were hardy to −13.5°, indicating that some increase in hardiness was obtained by photoperiod alone. The non-dormant, hardened condition was obtained by placing plants

Table I. *Development of Cold Hardiness in* Viburnum plicatum tomentosum *as Influenced by Photoperiod and Temperature*

These values were measured 12 weeks from beginning of treatments.

Plant location	Photoperiod	Dormant	Killing point °*
Greenhouse	Long	No	−6.0 a
Greenhouse	Short	Yes	−13.5 b
Outdoors	Long	No	−27.2 c
Outdoors	Natural (Short)	Yes	−29.6 d

* Killing points followed by identical letters are not significantly different at the 0.05 probability level.

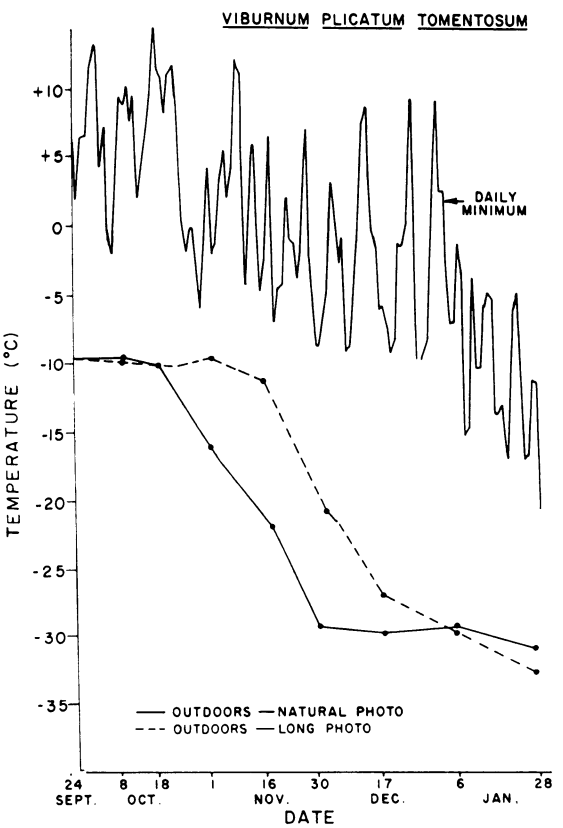

FIG. 1. Development of hardiness of *Viburnum* under natural and long photoperiods during the fall. Minimum daily temperatures are shown.

under natural fall conditions and extending the photoperiod. Surprisingly, hardiness by December 15 developed to −27.2° under these long days with natural temperatures. Proof that these plants were not dormant was obtained by taking a separate group into a warm greenhouse under LD where growth resumed almost immediately. Under the assumption of the dormancy requirement theory, these plants should have failed to harden when exposed to a treatment that prevented dormancy development. However, their hardiness was almost identical to plants located outdoors under natural photoperiods (the dormant, hardened plants), which hardened to −29.6°.

Hardiness Development Under Artificial Conditions Without Dormancy. The information in table I is consistent with data obtained from an experiment where hardiness was determined at 2-week intervals on plants placed outdoors under natural photoperiods or LD during the autumn of 1965 (fig 1). The development of hardiness in conjunction with minimum temperatures during this time provides additional insight into why LD treated (non-dormant) plants developed hardiness. Under natural conditions a large increase in hardiness was produced between October 18 and November 30, while the non-dormant, LD plants made a similar increase between November 16 and December 17. Although the development of hardiness was delayed by about 1 month, it was by no means prevented.

What brought about hardiness under LD conditions? The most likely explanation is that even though the plants under LD continued to grow for a period in the fall, growth had ceased by November 1 and by late November the night temperature dropped below −4° on 3 successive nights. These cold temperatures killed the foliage but did not affect the stem tissue. Removal of the leaves that perceived the LD influence quite likely was critical for subsequent hardening. It therefore seems possible that under long days and normal temperatures a hardiness inhibitor was produced in the leaves which prevented hardening. Thus, killing the leaves by low temperature removed this inhibition.

An experiment was also conducted in a cold chamber at 5° under LD (16 hrs) in an attempt to induce hardiness without dormancy under controlled conditions. After 28 days under LD at 5°, 1 group of each species remained under the same conditions while another group was transferred daily for 6 hours during the dark period to a freezer, which was lowered 0.5° per day, beginning at 1° and dropping to −6° in 14 days. This treatment is referred to as the low temperature sequence in table II.

Table III. *Induction of Cold Hardiness in* Acer negundo *by Short Days or Long Days and 5° Night Temperatures*

Treatment*	Killing point °**
Short days	—31.1 a
Long days + 5° night temperatures	—29.2 a
Long days	—18.5 b

* Subjected to 4 weeks under the respective photoperiods followed by 4 weeks of hardening in darkness at 5°.

** Killing points followed by identical letters are not significantly different at the 0.05 probability level.

Considerable development of hardiness was obtained with LD and low temperatures (table II). The *Viburnum* plants given the low temperature sequence were hardy to −26° while those receiving LD at 5° failed to survive −17°. The *Acer* plants given the low temperature sequence were hardy to −31.7° compared to −19.5° for those receiving 5° continuously. None of the treatments induced dormancy.

Alteration of the Long Photoperiod Response by Low Temperature. Having accomplished the development of substantial levels of hardiness by LD at 5° with the low temperature sequence, without inducing dormancy as a prerequisite, the next step was to determine if 5° during the 8-hour night would induce hardiness. *Acer* plants were subjected to SD (8 hrs) and LD (16 hrs) treatments while a third group received LD plus 5° at night for 4 weeks. All treatments were subsequently hardened in darkness at 5° for 4 weeks, frozen, and the killing points determined (table III). The SD control group was killed at −31.1° while the LD control was killed at −18.5°. The LD plus 5° night treatment did develop hardiness, to −29.2°, a figure not significantly different from the SD control. Here again, hardiness was induced in sizeable levels without dormancy being developed.

If low temperature given continuously or during

Table II. *Development of Cold Hardiness in* Viburnum *and* Acer *Under Long Photoperiods and Low Temperature Treatments*

Plant species	Continuous 5° 6 weeks	Dormant	Killing point °**
Viburnum	Temperature treatment*	No	—17.0 a
	Low temperature sequence	No	—26.1 b
Acer	Continuous 5° 6 weeks	No	—19.5 a
	Low temperature sequence	No	—31.7 b

* Low temperature sequence was 4 weeks at 5° under long days; then +1° to —6° in 14 days (lowered 0.5°/day) during dark period only.

** Killing points followed by identical letters are not significantly different at the 0.05 probability level.

the 8-hour dark period could bring about an increase in hardiness under LD, the next step was to consider whether or not a particular 4-hour cold period during the night would increase hardiness. An experiment using *Acer* plants was designed to test the effect of cold periods during the night on hardiness. In addition, groups of plants were placed in LD, SD, and LD plus 5° night temperatures but were given no subsequent hardening in order to determine if the 5° temperature itself had a hardening effect during the photoperiodic preconditioning treatment (table IV).

The low temperatures given during LD were effective in bringing about hardiness. However, there was no difference as a result of cold treatment given during the first or second halves of the dark period. Unfortunately, the effect of low temperature given during different parts of the light period was not tested. Failure to detect a difference in response to cold exposure given during the first or last half of the night may be due to the confounding effects of low temperature on hardening rather than to photoperiod itself. Additional work in this area is needed to separate and clarify the influence of low temperature under these conditions.

There was no difference between LD and LD + 5° night temperatures if the plants were not hardened, indicating that the low night temperature effect was realized only during the hardening treatment. This was the case even though growth was somewhat retarded by the 5° night temperatures during this time. In addition, the low temperature exposure was incapable of bringing about the dormant condition after 4 weeks under these LD conditions.

To further explore the possibility of producing hardiness without induction of dormancy, plants from each species were placed under LD at 10° (a non-hardening temperature), SD at 10°, or SD at 21° during the preconditioning period of either 4 or 5 weeks. At the end of this period, one-half of the plants were defoliated and placed in the greenhouse under LD to determine dormancy, and the other half were subjected to hardening temperatures in darkness. Table V shows that *Acer* survived −29° regardless of treatment, which is in contrast to the usual −15° to −17° killing point obtained by similar hardening after LD at 21°. In addition, *Viburnum* plants exposed to LD at 10° were killed at −22.9°, nearly 10° greater than the average killing point with LD at 21°. Yet, dormancy was not induced by the LD at 10°. Nevertheless, SD at 10° was effective in bringing about the dormant condition and a sizeable degree of hardiness in contrast to the effects reported by Moshkov (7).

Table IV. *Effect of Photoperiod and Low Temperatures During the Dark Period on Hardiness of* Acer negundo

Treatment*	Hardening**	Killing point °***
Short days	Yes	−31.1 a
Long days + 5° night	Yes	−29.2 a b
Long days + 5° night 2nd half of dark period + 4 more hrs during light	Yes	−27.0 b
Long days + 5° night 1st half of dark period + 4 more hrs during light	Yes	−26.3 b
Long days	Yes	−18.5 c
Short days	No	−12.2 d
Long days	No	−8.4 e
Long days + 5° night	No	−8.5 e

* Subjected to 4 weeks under the respective photoperiods.

** Four weeks of hardening in darkness at 5°.

*** Killing points followed by identical letters are not significantly different at the 0.05 probability level.

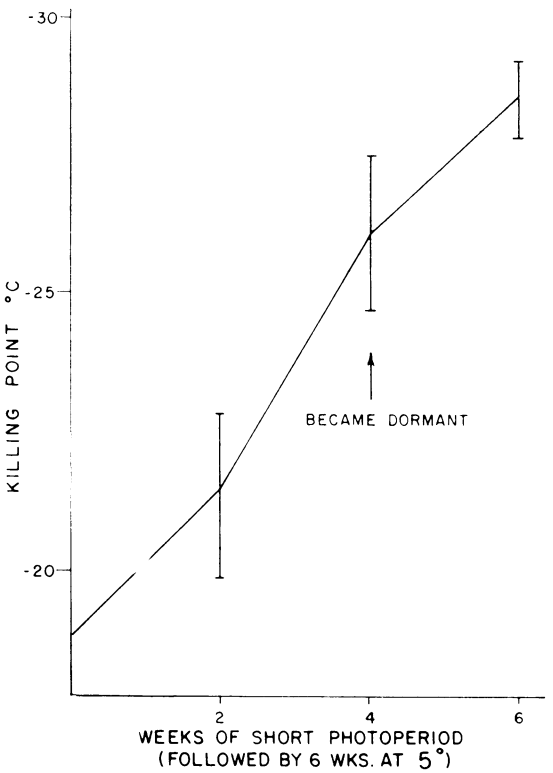

FIG. 2. Effect of short days on hardiness of *Acer negundo*. Standard deviations are shown.

Obtaining such hardiness indicates that the 10° temperature during the preconditioning period forced the plant to respond physiologically more like it was exposed to SD than to LD. Apparently, the 10° temperature altered the plant's ability to respond to LD. However, the fact that the LD 10° treatment failed to produce the dormant condition in either species illustrates that there was not complete reversal of the photoperiodic influence by low temperature.

Induction of Hardiness by Short Days. If hardiness development is not dependent on dormancy, what triggers the reactions that bring about hardiness increases of over 30° in many plants? To investigate

Table V. *Effect of 10° Temperature Prior to Hardening on the Subsequent Development of Hardiness of* Acer *and* Viburnum

Species	Treatment	Dormant	Killing point °*
Acer	Long days at 10°, 4 weeks	No	—29.0 a
	Short days at 10°, 4 weeks	Yes	—29.0 a
	Short days at 21°, 4 weeks	Yes	—29.0 a
Viburnum	Long days at 10°, 5 weeks	No	—22.9 a
	Short days at 10°, 5 weeks	Yes	—28.8 b
	Short days at 21°, 5 weeks	Yes	—30.2 b

* Killing points followed by identical letters are not significantly different at the 0.05 probability level.

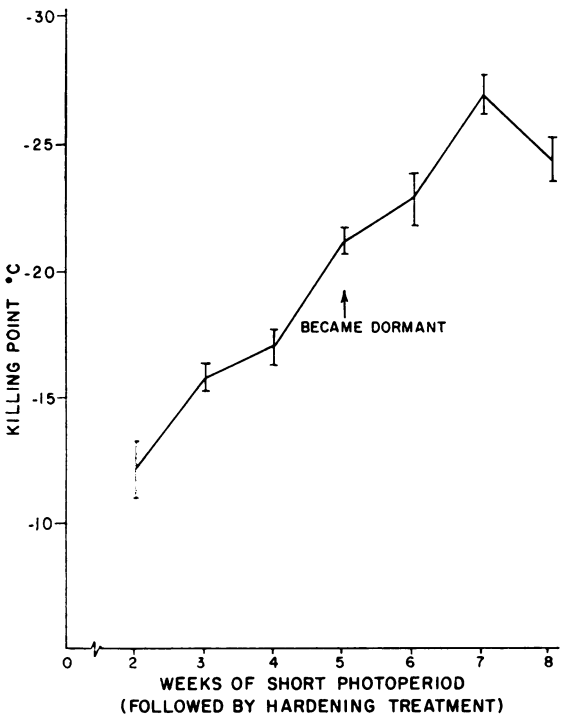

FIG. 3. Effect of short days on hardiness of *Viburnum*. Standard deviations are shown.

this, experiments were conducted to determine if cold hardiness induction was in response to short days. *Acer* and *Viburnum* were exposed to varying periods of short days, followed by a hardening period in darkness at 5° or lower. Figures 2 and 3 show that with both species hardiness increased in a linear fashion in response to increasing exposure to SD. Hardiness of *Acer* increased steadily over the 6-week induction period going from about −19° with no SD to −28.5° with 6 weeks of such treatment. Hardiness of *Viburnum* increased linearly from 2 to 7 weeks of SD, ranging from −12° with 2 weeks to −26.7° after 7 weeks. If dormancy per se were required before hardening could proceed, no gain in hardiness would occur for about 4 weeks with *Acer* and 5 with *Viburnum*, which is the time required for the induction of dormancy. Instead, hardiness increased steadily for 6 and 7 weeks.

Table VI gives an indication of the effect of SD upon subsequent hardiness developed. At the end of the 6-week photoperiod treatment, but prior to low temperature exposure, SD had increased hardiness slightly (2.6°). However, after 4 weeks of hardening, the SD influence had widened the difference to nearly 14°. The LD treatment gained about 8° during hardening while SD gained nearly 19°, indicating that the rate of hardening after SD exposure was twice as rapid as after LD treatment.

The response of *Acer* to 5° temperature in darkness after having been exposed to 0, 2, 4, and 6 weeks of SD is shown in figure 4. There is a progressive increase in hardening by all 4 treatments in response to 5° temperatures. However, the slopes of the lines are distinctly different; the greater the number of SD, the steeper the slope. For instance, there was a 4° difference between the 0- and 6-week SD treatments at the outset and nearly 11° after the hardening period.

To further verify that the SD influence was truly a photoperiodic phenomenon, the night interruption experiment was conducted. Plants were given either a 9-hour SD, a 16-hour LD, or an 8-hour SD plus 1-hour night interruption in the middle of the 16-hour dark phase. In accordance with the SD concept of hardiness, the night interruption treatment produced hardiness almost identical to LD (table VII). Those plants receiving LD or SD and interrupted nights were killed at near −18° while the SD treatment was hardy to −31°. Table VIII gives an indication of the ready reversibility of the effect of SD. While 4 weeks of SD increased the hardiness level 12° from −15.5° to −27.2° and 2 weeks of SD lowered the

Table VI. *Effect of Photoperiod on Level of Hardening of* Acer

Treatment	Hardening*	Killing point °**
Long days, 6 weeks	No	—6.5 a
Short days, 6 weeks	No	—9.1 a
Long days, 6 weeks	Yes	—14.8 b
Short days, 6 weeks	Yes	—28.0 c

* Four weeks at 5° in darkness.

** Killing points followed by identical letters are not significantly different at the 0.05 probability level.

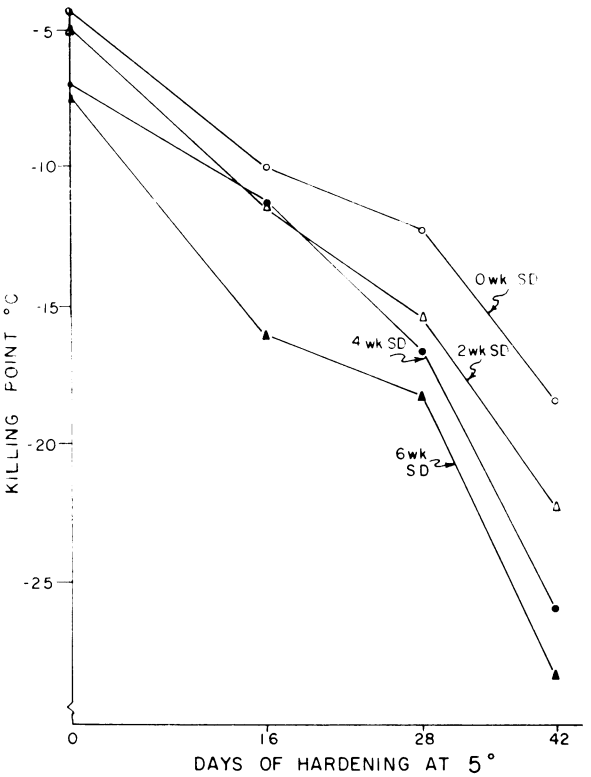

FIG. 4. Effect of photoperiod and progressive hardening on hardiness of *Acer*.

Table VII. *Effect of Night Interruption and Other Photoperiodic Treatments on the Induction of Hardiness of* Acer negundo

Treatment*	Killing point °**
Short days	—31.1 a
Short days + 1 hr night interruption	—18.3 b
Long days	—18.5 b

* Subjected to 4 weeks under the respective photoperiods followed by a hardening period of 4 weeks at 5°.

** Killing points followed by identical letters are not significantly different at the 0.05 probability level.

Table VIII. *Reversibility of the Photoperiod Effect on Hardiness of* Acer negundo

Treatment*	Killing point °**
Short days, 4 weeks	—27.2 a
Long days, 2 weeks; short days, 2 weeks	—19.5 b
Short days, 2 weeks; long days, 2 weeks	—15.5 c
Long days, 4 weeks	—15.6 c

* Subjected to 2 or 4 weeks of the respective photoperiods followed by a hardening period of 4 weeks at 5°.

** Killing points followed by identical letters are not significantly different at the 0.05 probability level.

killing point by 4°, the succeeding 2 weeks of LD completely cancelled the 4° increase. This ability to rapidly reverse the effect of SD with long photoperiods provides evidence that hardiness induction is somewhat unstable, at least during the initial stages.

Thus, the data indicate that bud dormancy and cold hardiness development are distinctly separate and independent and that both SD with subsequent low temperature or LD with proper low temperature will develop hardiness in woody species.

As shown in table I, the effects of dormancy (induced by photoperiod) and low temperature are additive rather than synergistic. The combined effects of dormancy without hardening (7.5°) and hardening without dormancy (21.2°) increased hardiness over 28°, while the combination taking both into account (dormant: hardened) resulted in only a 23° increase in hardiness.

It has been a mistake to assume that dormancy was a necessary prerequisite for hardening, simply because they occurred in that order during the same season. Photoperiod-induced dormancy, without low temperature, resulted in small increases of 3 to 6° hardiness, which could account for earlier observations, such as those of Kramer (5), that dormant material was somewhat less susceptible to cold periods in early fall. However, this condition is likely a result of short days and not dormancy per se. Cold hardiness development in woody plants thus appears to be a photoperiodic phenomenon similar to other processes such as flowering, tuberization, and dormancy induction.

Literature Cited

1. CHANDLER, W. H. 1954. Cold resistance in horticultural plants: A review. Proc. Am. Soc. Hort. Sci. 64: 552–72.
2. DAVIDSON, H. AND C. L. HAMNER. 1957. Photoperiodic response of selected woody ornamental shrubs. Quart. Bull. Mich. Agri. Expt. Sta. 40: 327–43.
3. HOWARD, W. L. 1910. An experimental study of the rest period in plants. Univ. Mo. Agri. Expt. Sta. Bull. No. 1.
4. IVANOV, J. M. 1935. Frost resistance of citrus plants as controlled by daylength. Compt. Rend. Acad. Sci. URSS 27: 736–38.
5. KRAMER, P. J. 1937. Photoperiodic stimulation of growth by artificial light as a cause of winter killing. Plant Physiol. 12: 881–83.
6. LE CLERG, E. L., W. H. LEONARD, AND A. G. CLARK. 1962. Field Plot Technique. Burgess and Company.
7. MOSHKOV, B. S. 1935. Photoperiodismus und frostharte ausdauernder Gewachse. Planta: 23: 772–803.
8. STEPONKUS, P. L. 1967. The role of light in cold acclimation of *Hedera helix* 'Thorndale'. Ph.D. Thesis. Purdue University, Lafayette, Indiana.
9. VAN DER VEEN, R. AND G. MEYER. 1959. Light and Plant Growth. MacMillan, New York.