Plant Physiol. (1968) 43, 9–13

Regulation of Cold Hardiness in Acer negundo[1, 2]

R. M. Irving[3] and F. O. Lanphear
Department of Horticulture, Purdue University, Lafayette, Indiana

Received July 26, 1967.

Abstract. **The application of gibberellin to *Acer negundo* either during or after a short photoperiod strikingly lowered the amount of hardiness obtained after 4 weeks in darkness at 5°. Two growth retardants, B9 and Amo 1618, the latter of which interferes with gibberellin synthesis, brought about hardiness increases under long photoperiods. The naturally occurring inhibitor, dormin, also increased hardiness under the usual inhibiting influence of long photoperiods. Extracts from plants given long or short days had gibberellin-like compounds in largest quantities during LD and lowest quantities under SD, while the inverse was true for the inhibitor.**

It has been shown that cold hardiness induction in woody plants is a photoperiodic phenomenon (5). Short days (SD) induce hardiness while long days (LD) are inhibitory. In addition, it has been shown that LD treated leaves on a plant can retard the SD promoting effect of other leaves on the same plant (6). Thus, the perception of the long photoperiod allows the production of substances inhibitory to hardening. Exposing branched plants to long and short photoperiods indicated there was a transfer and balance of growth and hardiness regulating compounds from 1 branch to another (6). Since the compound(s) were readily translocated and produced in largest quantities during long days, a logical suspect was gibberellin. Thus, the influence of gibberellin and various growth retardants on cold hardiness development was investigated.

Methods and Materials

Plant Material and Experimental Conditions. *Acer negundo* L. (Box elder) seedlings were used as test plants for hardiness determination. Seedlings were grown for at least 3 months at approximately 21° in a greenhouse under long photoperiods before undergoing experimental conditions in controlled environment chambers.

A completely randomized design was used and the analysis of variance was performed according to the procedure of Le Clerg *et al.* (7) on the individual killing points to determine which variables were significant. Duncan's new multiple range test was utilized for mean separation of the hardiness levels of all experiments.

Artificial Freezing Test and Determination of Viability. 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 in the freezing tests. A 16 cm section from each particular plant was cut into 6 pieces and each was exposed to different temperatures. One section at 5° served as the control. The others were placed in styrofoam boxes and then in a freezer at −6.5°. The rate of temperature drop was less than 3°/hour. Air temperatures were monitored in each box at 2 and one-half minute intervals and automatically recorded. When the temperature in the boxes reached −5°, all the boxes except 1 were transferred to a freezer set at −12.5°. This process was repeated at −17.8, −23.5, and −29.0°. After 2 hours at each temperature, the boxes were removed and allowed to thaw at 5° for 6 hours. The samples were then placed in a plastic container under high humidity at room temperature for 36 hours.

Determination of the viability of the frozen tissue and the extrapolation of the killing points were performed by using a slight modification of the triphenyl tetrazolium chloride (TTC) technique as previously outlined (5, 8).

Extraction Techniques. The extraction technique employed to remove gibberellins and inhibitors was similar to that used by Eagles and Wareing (3). In each case, the material to be extracted was shredded with a razor blade and then homogenized in a blender at 0° in 80% (v/v) aqueous methanol. Three successive changes of methanol were made at 8-hour intervals. The fractions were filtered through Whatman No. 42 filter paper and then concentrated to 100 ml under reduced pressure at 28°. The aqueous fraction was allowed to set overnight, refiltered, and acidified to pH 2.0 with

---

[1] Journal Paper No. 3118, Agricultural Experiment Station, Purdue University, Lafayette, Indiana.

[2] This study represents a portion of a thesis presented to the faculty of the Graduate School of Purdue University in partial fulfillment for the Ph.D. degree.

[3] Present address: Department of Horticulture, Oklahoma State University, Stillwater, Oklahoma.

5 % H_2SO_4. This fraction was then partitioned 8 times with 40 ml of ether and the ether fraction reduced to dryness under reduced pressure and dissolved in a known volume of dry ether for loading on chromatograms.

Paper Chromatography. Concentrated extracts were strip-loaded on Whatman No. 1 MM chromatography paper and the chromatograms developed by the ascending method until the solvent front was 25 cm from the starting line. The chromatograms were air dried and cut into 10 transverse strips for bioassay using the lettuce germination test.

Dark Germination of Lettuce Seed Bioassay. This test was based on the 1 described by Harada (4). Chromatograms were cut into 10 sections as mentioned above. Each section was then placed in a Petri dish and moistened with 1.3 ml of distilled water. (When GA or dormin solutions were used, 1.3 ml of the particular concentration desired was placed on a blank chromatogram strip). One hundred lettuce seeds, variety Grand Rapids, were then sown on each moistened strip. The dishes were placed in the dark at 25° and the percent germination determined after 2 days.

Chemicals. The gibberellic acid (GA) used was the 10 % salt of GA_3 obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. The dormin was obtained from Dr. J. Cherry through the courtesy of Dr. J. van Overbeek, Shell Development Company, Modesto, California. The Amo 1618, chemically known as 4-hydroxy-5-isopropyl-2methylphenyl trimethyl-ammonium chloride, 1-piperidine carboxylate, was obtained from E. C. Geiger Company, North Wales, Pennsylvania, and the B9 (Alar or B995), chemically known as N-dimethylaminosuccinamic acid, was received from Naugatuck Chemical Corporation, Naugatuck, Connecticut.

Results

Effects of Gibberellin and Growth Retardants Given During the Induction Period on Growth and Hardiness. The possibility that SD were bringing about hardening by lowering or overcoming gibberellin activity was investigated by dividing 15 *Acer* plants into 3 groups. One group was placed under LD and sprayed at weekly intervals with 3000 mg/l concentration of B9, a growth retardant; the second group was placed under LD; the third group under SD. The latter 2 treatments were sprayed with water at weekly intervals.

The stem growth of the plants in the 3 treatments was measured in order to determine the effectiveness of the inhibitor application. The plants grown under LD continued to grow quite rapidly and produced an increase of 42 mm in length during the 4-week treatment period (fig 1). The B9 applications were quite effective in limiting the growth of *Acer,* allowing only 7 mm increase in 4 weeks. The SD treatment allowed only an average of 5 mm growth.

The plants were subsequently hardened for 4 weeks at 5° in darkness and the killing points determined. As shown by the numbers in parenthesis in figure 1, B9 was also effective in bringing about an increase in hardiness. Long days brought about hardiness to −16.1°, while LD + B9 was killed at nearly −20.0°, an increase of 4°. However, the SD treatment was still more effective, the killing point being −22.2°.

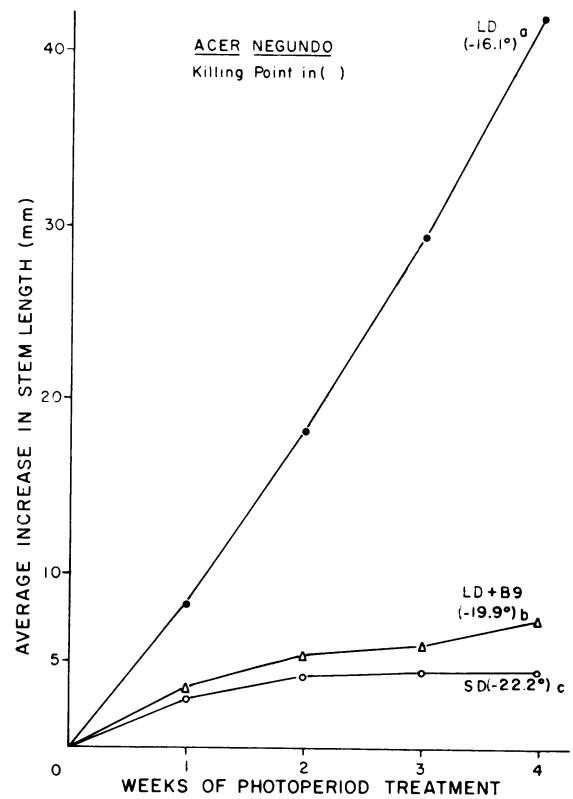

FIG. 1. Effect of B9 on the growth and hardiness of *Acer.* B9 was applied weekly at 3000 mg/l. Killing points followed by identical letters are considered not significantly different at the 0.05 probability level.

The influence of GA and Amo 1618, a growth retarding compound shown to inhibit gibberellin synthesis (2), on the growth and hardiness of *Acer* was also determined. Plants were placed under the following conditions: 1) SD; 2) LD; 3) SD + a 500 mg/l spray application of GA each week; 4) LD + a 1000 mg/l spray application of Amo 1618 each week; and 5) LD + a spray application of 25 mg/l of GA and 1000 mg/l of Amo 1618 each week.

Gibberellin treated plants produced a tremendous burst of growth even under SD, growing an average of 96 mm compared to 59 mm produced by LD

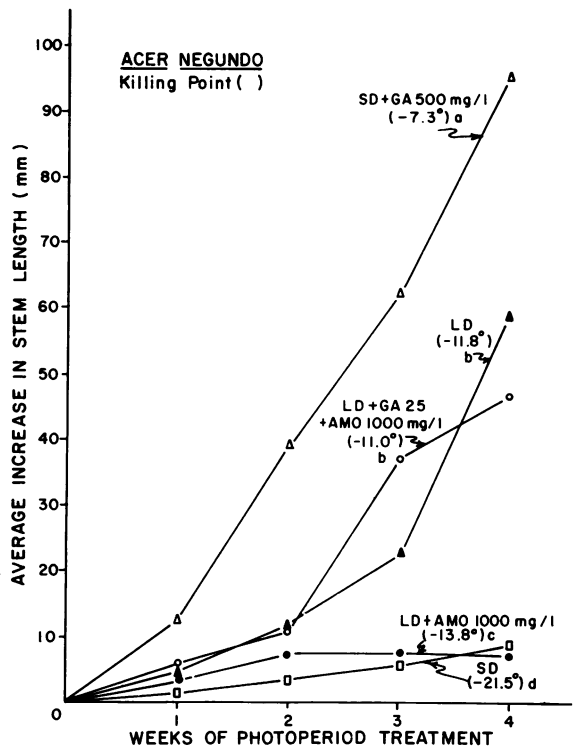

FIG. 2. Effect of gibberellic acid and Amo 1618 on the growth and hardiness of *Acer*. Numbers following the chemical abbreviation indicate concentration in mg/l. Killing points followed by identical letters are considered not significantly different at the 0.05 probability level.

during the same period (fig 2). The plants treated with both GA and Amo 1618 grew somewhat less (46 mm) than LD treated plants, while the LD + Amo 1618 treatments both grew less than 10 mm. A separate group of SD + GA treated plants was defoliated and moved to a warm greenhouse under LD and compared with plants treated with SD (and no GA) to determine if they were dormant. Only those treated with GA put out new foliage, indicating that the exogenous applications had prevented the induction of dormancy by SD.

The plants were subsequently hardened at 5° for 4 weeks before determining the killing points. The plants treated with GA under SD did not harden at all and were even 4° less hardy than LD. The growth retardant, Amo 1618, did lower the killing point under LD, from −11.8° to near −14.0°. This treatment was repeated 3 times and in each case the Amo 1618 produced a relatively small (3-4°), but significant, increase in hardiness. The treatment receiving both LD + Amo 1618 and GA hardened much like LD. Thus, the GA canceled the hardiness promoting effect of Amo 1618.

Since weekly applications of B9 and Amo 1618 did bring about modest increases in hardiness, higher concentrations and more frequent applications were attempted in order to more nearly approximate the SD effect. Concentrations of 2000 mg/l of Amo 1618 brought about visible chlorosis of *Acer* after 1 application; therefore, 1000 mg/l applied 3 times weekly was used. In addition, B9 at 3000 mg/l was applied 5 times weekly to a second group of plants under LD. The effects of these multiple applications were quite marked (table I). The administration of Amo 1618 brought about an increase of 10° after 4 weeks of hardening at 5° while the B9 applications produced an 8° increase.

Table I. *The Influence of Spray Applications of Amo 1618 and B9 on Hardiness of* Acer negundo

Treatment[1]	Killing point °[2]
SD 5 wks	—29.6 a
LD + Amo 1618 (Applied at 1000 mg/l 3 times weekly for 5 wks)	—27.9 a
LD + B9 (Applied at 3000 mg/l 5 times weekly for 4 wks)	—26.4 b
LD 5 wks	—17.6 c

[1] Subjected to 4 and 5 weeks under the respective photoperiods followed by 4 weeks of hardening at 5°.
[2] Killing points followed by identical letters are considered not significantly different at the 0.05 probability level.

Effect of GA Applied After the Induction Period. In order to determine if GA was also able to prevent hardiness after SD induction and prior to hardening, plants previously given 6 weeks of SD were immersed in a 1000 mg/l solution of GA for 2 minutes 2, 1 and 0 days before beginning a 6-week hardening treatment at 5°. The GA treatments were all effective in significantly lowering the hardiness level (table II). The application given 2 days before hardening was, indeed, most effective in reducing hardiness. However, the GA

Table II. *Effect of GA Applied at Different Time Subsequent to Short Days and Prior Hardening of* Acer negundo

Treatment[1]	Killing point °[2] Expt 1	Expt 2
SD (No GA)	—39.4 a	—33.3 a
SD + GA 1000 mg/l (Applied when hardening began)	—34.5 b	—28.2 b
SD + GA 1000 mg/l (Applied 1 day before hardening began)	—35.0 b	—27.9 b
SD + GA 1000 mg/l (Applied 2 days before hardening began)	—33.7 c	—26.5 c

[1] Subjected to 6 weeks under the respective photoperiods followed by 6 weeks of hardening at 5°.
[2] Killing points followed by identical letters are considered not significantly different at the 0.05 probability level.

treatments were not able to completely eliminate the SD influence.

The ability of GA to prevent the development of cold hardiness when applied after LD + 5° night temperature induction was tested in a similar fashion (table III). The GA treatment lowered the hardiness level 9 degrees from −29° to −20°, a temperature nearer the LD than the SD treatment. If the amount of hardiness loss as a result of GA application in this experiment is compared with the largest losses brought about by GA after SD exposure (5.7° and 6.8° shown in table II) the differences are not large. However, the relative degree of hardiness lost by GA application to the LD + 5° night treated plants was much greater than in the case of SD.

Table III. *Effect of Low Temperatures During the Dark Period and GA Given Before Hardening on Hardiness of* Acer negundo

Treatment[1]	Killing point °[2]
SD	—31.2 a
LD + 5° night temp	—29.1 a
LD + 5° night temp + GA 1000 mg/l 2 days before hardening	—20.1 b
LD	—18.5 b

[1] Subjected to 4 weeks under the respective photoperiods followed by 4 weeks of hardening at 5°.

[2] Killing points followed by identical letters are considered not significantly different at the 0.05 probability level.

The recently identified hormone, dormin, has also been shown to be a gibberellin antagonist (1, 9). Dormin showed an interaction with GA in the oat coleoptile test and inhibited GA-stimulated synthesis of α-amylase in barley endosperm, indicating that it functions as an anti-gibberellin in vivo. In addition, it has been shown to retard the stem extension of birch plants and inhibited bud break in isolated segments of birch stems. Thus, the effect of dormin was tested on the growth and hardiness of *Acer*. The chemical was applied during LD by 2 methods: (1) by allowing the plant to continuously take it up through a leaf folded into a vial containing a 100 mg/l solution of dormin; and (2) by painting the leaves every other day with a 100 mg/l solution of dormin. After 3 weeks of treatment, the plants were hardened for 4 weeks in darkness at 5°.

The dormin was as effective as SD in suppressing the growth of *Acer* plants. Similarly, the dormin treatments increased hardiness by a substantial margin (fig 3). The SD treated plants were killed at −28.5° and the LD + dormin (fed through vials) were hardy to −21.1°, over 6° lower than without dormin. Dormin applied by painting the leaves also significantly increased hardiness, but less than that applied through vials.

Bioassay of Hardiness Inhibitors and Promoters. Since dormin and other growth retardants increased

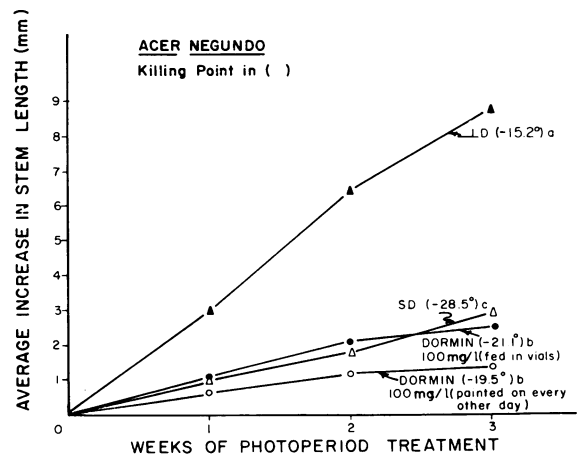

FIG. 3. Effect of dormin on the growth and hardiness of *Acer*. Killing points followed by identical letters are considered not significantly different at the 0.05 probability level.

hardiness while GA decreased it, an attempt was made to determine if hardiness development were related to a counter-action of GA by an inhibitor, such as dormin, under SD. Determination of the levels of gibberellin and inhibitor under both LD and SD was particularly important since previous data indicated that gibberellin prevented hardiness development while inhibitors increased it. Thus, extractions of large volumes of SD and LD treated leaves were made, chromatographed and compared in the lettuce germination test to a solution containing 25 mg/l each of GA and dormin. N-Butanol:1.5 N NH_3 at 3:1 was used as the solvent because it brought about a wide resolution of GA and dormin.

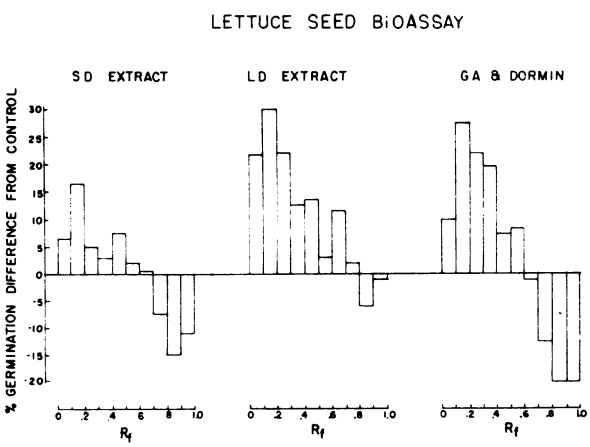

FIG. 4. Comparison of LD and SD extracts with dormin and GA on germination of lettuce seed. The values for the plant extracts are the average of 4 separate assays of 12 g fresh weight in each. The dormin and GA values are the average of 2 assays of 25 mg/l of each. The solvent was *n*-Butanol: 1.5 N NH_3 (3/1 v/v).

The relative levels of gibberellin-like compounds were higher under LD than with SD (fig 4). Likewise, the amount of inhibitor was increased by SD treatment. The germination-promoting areas in the 2 extractions were very similar to the peak produced by GA, all 3 being located primarily between R_F 0.0 and 0.4. Correspondingly, the inhibiting zones of the extracts were very similar to the inhibition produced by dormin. The *Acer* SD extract contained the greater amount of inhibitory activity and, therefore, resembled the activity of dormin quite closely.

Discussion

Work conducted on *Acer negundo* plants demonstrates that gibberellin acts as an inhibitor to cold hardiness induction. Applications of GA produce large amounts of growth, even under SD, and eliminate the usual hardiness obtained by SD. Three compounds which exhibit anti-gibberellin or growth retarding properties (B9, Amo 1618, and Dormin) were effective in inducing measurable levels of hardiness under LD, presumably by counteracting the influence of endogenous gibberellin. This counteraction of gibberellin apparently simulates the SD effect which normally precedes hardening.

In addition, administration of GA subsequent to the SD preconditioning period and prior to hardening in darkness significantly lowered, but did not completely eliminate, the hardiness levels attained. GA given after LD + 5° nights and before the hardening treatment prevented hardening in the usual manner. Why GA did not completely remove the hardiness induced by SD, but did under LD with 5° nights is not clear, but one could speculate that the presence of an inhibitor produced under SD, but not under LD + 5° nights, might partly counteract the GA activity. Once the SD induction has occurred it may take more than GA to overcome it.

An inhibitor extracted from SD treated *Acer* leaves, using techniques similar to extraction of dormin in other species, brings about inhibition similar to dormin in the lettuce germination test. Extraction of large volumes of leaves previously exposed to SD showed relatively lower levels of gibberellin-like compounds and higher levels of inhibitor than LD treated leaves. These results are consistent with the idea that long days inhibit hardiness as a result of high gibberellin activity and short days promote hardiness by the buildup of an inhibitor able to counteract gibberellin.

Literature Cited

1. CHRISPEELS, M. J. AND J. E. VARNER. 1967. Hormonal control of enzyme synthesis: on the mode of action of gibberellic acid and abscisin in aleurone layers of barley. Plant Physiol. 42: 1008–16.
2. DENNIS, D. T., C. T. UPPER, AND C. A. WEST. 1965. An enzymatic site of inhibition of gibberellin biosynthesis by Amo 1618 and other plant growth retardants. Plant Physiol. 40: 948–52.
3. EAGLES, C. F. AND P. F. WAREING. 1964. The role of growth substances in the regulation of bud dormancy. Physiol. Plantarum 17: 697–709.
4. HARADA, H. 1962. Etude des substances naturelles de croissance en relation avec la floraison. Isolement d'une dubstance de montaison. Rev. Gen. Botan. 69: 201–97.
5. IRVING, R. M. AND F. O. LANPHEAR. 1967. Environmental control of cold hardiness in woody plants. Plant Physiol. 42: 1191–96.
6. IRVING, R. M. AND F. O. LANPHEAR. 1967. The long day leaf as a source of cold hardiness inhibitors. Plant Physiol. 42: 1384–88.
7. LECLERG, E. L., W. H. LEONARD, AND A. G. CLARK. 1962. Field Plot Technique. Burgess and Company.
8. STEPONKUS, P. L. AND F. O. LANPHEAR. 1967. The refinement of the tripenyl tetrazolium chloride method of determining cold injury. Plant Physiol. 42: 1423–26.
9. THOMAS, T. H., P. F. WAREING, AND P. M. ROBINSON. 1965. Action of the sycamore "Dormin" as a gibberellic antagonist. Nature 205: 1270–72.