Changes in Endogenous Growth Substances in the Embryos of *Juglans regia* During Stratification[1]

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Abstract. Kernels from *Juglans regia* walnuts stratified at 0°C were sampled at weekly intervals and extracted with methanol. The extracts were partitioned into 4 phases which were water, neutral ether, acidic ether and acidic butanol, then bioassayed for cytokinins, gibberellins, auxins and inhibitors. No cytokinins nor gibberellins were found in the tissue. There was activity analogous to that from auxins. An inhibitor which diminished during stratification was found. This inhibitor is believed to be abscisic acid, on the basis of UV absorption spectrum, Rf values established by co-chromatography on paper and silica gel plates, and derivatives analyzed by gas liquid chromatography.

Introduction

IN most cases, deciduous fruit tree seeds are inherently protected from adverse environmental conditions such as low temperature by the state of dormancy. While protected from winter cold by dormancy the low temperatures play the important role of providing the requirement for winter chilling which in time breaks dormancy.

Though unproven, it appears plausible to ascribe the primary and directive mechanism of dormancy to the balance of growth promoting and inhibiting substances rather than to such factors as phosphate metabolism (20, 22) and respiration (12, 21, 24). The literature supporting the role of growth promoter and inhibitor balance in dormancy is voluminous (1, 2, 3, 8, 10, 23). The data from these works support the contention that the levels of promoters remain fairly constant while the levels of inhibitors decrease as the low temperature requirement is fulfilled. Also, the increase in endogenous gibberellins has been ascribed as a factor overcoming dormancy (6).

Naylor and Simpson (17) established that a gibberellin type seed component increased during the latter stages of dormancy. Others have shown that the application of gibberellin breaks rest in certain seeds (11). Yet another promoting factor, cytokinin, has been reported to break dormancy (16).

In order to regulate dormancy, more knowledge of its internal control mechanism is needed. The research reported in this paper was initiated to gain greater insight about the mechanism of dormancy by studying the changes in growth promoting and inhibiting compounds of walnut kernels during stratification.

Materials and Methods

Extraction. 'Payne' walnuts, *Juglans regia* L., with their hulls previously removed but unshelled, were stratified at 0°C in moist vermiculite. At weekly intervals, the nuts were taken out of the stratifying medium. The shells were removed and the fresh weight of the kernels was determined. The kernels were then cut into pieces and placed in a flask containing 100% methanol and stored at 0°C. After 4 days, the methanol was filtered off and replaced by 80% methanol; then the process was repeated on the 9th and the 14th day. The filtrates from each methanol change were combined and stored at 0°C. The alcohol insoluble substances (AIS) hereinafter called extracted dry weight, were dried in an oven maintained at 86°C. All calculations were made on the basis of the extracted dry weight (AIS).

A modification of Milborrow's (15) scheme, Fig. 1, was used for separating plant acids from the methanol extracts. The methanol extracts were concentrated to the aqueous phase under reduced pressure. The sample was centrifuged to remove any water insoluble debris and the supernatant decanted. Sodium bicarbonate at 5% was added to the water insoluble precipitate which was then triturated and centrifuged. The soluble fraction was decanted and stored for later analysis.

The aqueous supernatant was adjusted to pH 2.8 with dilute H_2SO_4. On adding NaCl to each sample, it was partitioned 3 times with ether. The ether phases were combined and concentrated to 50 ml. The aqueous-phase 1 was taken to dryness and stored in a freezer for later measurement of cytokinin.

The combined ether fraction was treated with 5% $NaHCO_3$ and water alternately. The aqueous and alkaline fractions were combined. The neutral ether-phase 2 (Fig. 1) containing the neutral and weakly acidic compounds was concentrated to dryness under reduced pressure and stored in a centrifuge tube in the freezer.

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would not appear promising as an apple thinning agent. On the other hand, it offers considerable promise as a treatment from prebloom to early postbloom stage where complete elimination of fruit is desired.

The loosening of the fruit at harvest as shown in Table 3 would be of particular value with cultivars to be harvested mechanically as pointed out previously (4). It would also benefit conventional hand harvesting where firm attachment slowed the operation and contributed to excessive removal of stems from the fruits or to broken spurs and bruising.

While the majority of these tests were conducted on unit branches rather than on entire trees the trials were duplicated under widely differing conditions and on a range of cultivars. Nevertheless, some variations are to be expected with applications made on entire trees.

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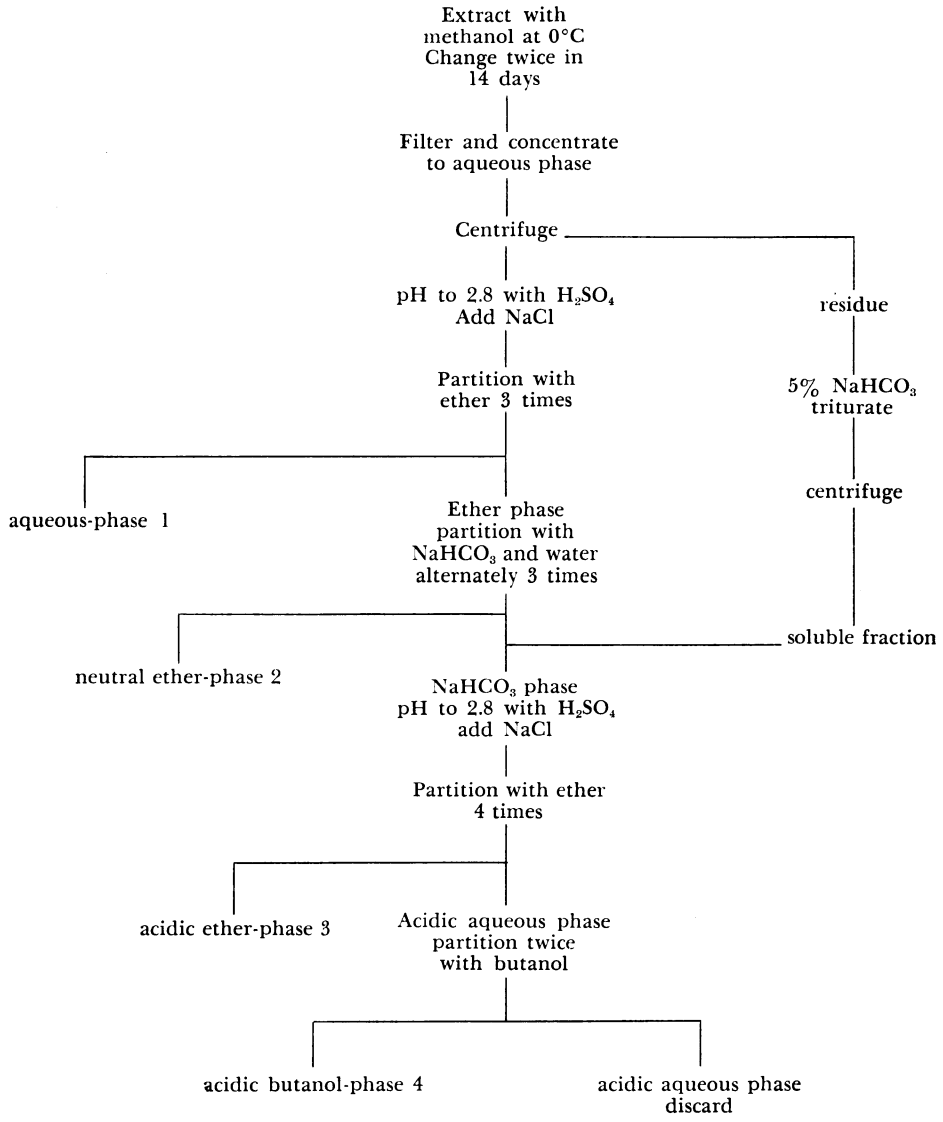

Fig. 1. Flow diagram for separation of walnut seed components.

The alkaline aqueous phase and the $NaHCO_3$ supernatant from the centrifugation of the cellular debris were combined. The solution was acidified to pH 2.8 with H_2SO_4 after which NaCl was added. The sample was partitioned 4 times with ether. The ether layers were combined and designated as acidic ether-phase 3. This fraction containing organic acids, indoles, etc., was also concentrated to dryness under reduced pressure in a centrifuge tube and stored in the freezer.

The acidified aqueous phase was saved and any ether present was blown off. It was then partitioned twice with butanol. The acidified aqueous phase was discarded while the acidic butanol-phase 4 was concentrated to dryness and stored in the freezer.

Lemna minor tests for cytokinins. Test tubes containing 25 ml of a modified Hoagland Nutrient Solution No. 1 (modified, as the copper was omitted) were sterilized for 20 min in an autoclave. Three *Lemna minor,* duckweed plantlets, each with 3 leaves, were transferred aseptically to each tube to be bioassayed. To some test tubes known amounts of abscisic acid and benzyl adenine were added and were included with the checks in each test. The aseptic cultures, placed on tilted trays covered with aluminum foil, were subjected to a mixture of fluorescent and incandescent lights in a growth control chamber maintained at 22–24°C and a 12-hr photoperiod. The cultures were allowed to grow for 9 days after which the plants were evaluated visually for growth.

The aqueous-phase 1 dissolved in water and the acidic ether-phase 3 dissolved in 100% methanol of the walnut kernel extract were evaluated by this test. Phase 1 and phase 3 were at 10 g extracted dry weight equivalent per ml and 20 g extracted dry weight equivalent per ml, respectively. Each fraction was tested at the rate of 5, 50 and 100 µl of extract per tube.

Wheat coleoptile test. For this test, the neutral ether-phase 2 and acidic ether-phase 3 samples were taken up in 100% methanol equivalent to 20 g extracted dry weight per ml. An aliquot of extract, e.g., 2 µl, containing 40 mg equivalent extracted dry weight, was spotted along the origin line on 3 MM chromatography paper. On evaporation of the methanol, the paper was irrigated with a solvent system of isopropanol:NH_4OH:H_2O (8:1:1 v/v/v). The strips were dried again and cut into 10 equal sections measuring 3 × 2 cm. These sections were assayed by the wheat coleoptile test (18) in duplicate and replicated 4 times.

Dwarf pea test. The bioassay was used to check for the presence of gibberellin-like substances in 2 extracted phases, the acidic ether-phase 3 and the acidic butanol-phase 4 and essentially followed the outline of Hayashi and Rappaport (9).

Thin layer chromatography (TLC). A known amount of each sample was applied on silica gel GF 254 plates and developed a distance of 10 cm using 2 solvent systems: 1) n-butanol; n-propanol; NH_4OH and H_2O, BPAW (2:6:1:2 v/v/v/v) and 2) benzene; methanol; formic acid (90%), BMF, (85:15:1.3 v/v/v). Though many other solvent systems were tested, their use was not found to be advantageous. Plates were developed twice in the solvents used, drying the plates between runs.

Gas liquid chromatography (GLC). Extractives for analysis were first separated by TLC as described above. The silica gel at given Rf values was scraped into a vial and the absorbed material eluted with 1% acetic acid in methanol. The eluate was evaporated to dryness and prepared for analysis according to methods described by Davis et al. (5) using bis (trimethylsilyl) acetamide (BSA) for silyl derivatives and MacMillan[2] using diazomethane for methylated derivatives.

Ultraviolet spectrophotometry. Components of the extractives separated by the TLC method described above were taken up in 0.005 N H_2SO_4 in 95% ethanol. The absorption spectrum for each constituent was determined in a Beckman DK–2 spectrophotometer. Standards were prepared in a like manner.

[2]MacMillan, J. 1968. Personal communication.

Results and Discussion

Walnut seeds when planted without stratification germinate poorly (Fig. 2) and the seedlings are characteristically dwarfed and atypical. The very few seeds that germinate after a week or less of stratification display 3 main growth forms: 1) a bare slender stem with no leaf development, 2) a stunted appearance with a meager rosette of leaves or 3) a stunted appearance with a single leaf. In no case do these plants assume normal growth. Though walnuts planted after 3 weeks' stratification develop seedlings normal in appearance, up to 7 weeks are required for seedling emergence. On the other hand, walnuts planted after 8 weeks' stratification emerge in 2 to 3 weeks.

Phase 1—aqueous. A concentration series of the aqueous extract shown in the flow diagram (Fig. 1) was applied to *Lemna minor* for detection of cytokinins. Based on the results from this test, there were no cytokinins present in the aqueous phase. No other test was conducted on the aqueous phase.

Phase 2—neutral ether. A concentration series of the neutral ether extract was assayed by the wheat coleoptile test. The amount of growth inhibition and promotion of coleoptiles treated with this extract was minute and insignificant. No additional test was made on this fraction.

Phase 3—acidic ether. The acidic ether fraction was bioassayed at 40 and 80 mg extracted dry weight equivalent using the wheat coleoptile test and at 40 to 200 mg extracted dry weight equivalent using the dwarf pea test and *Lemna minor*. Phase 3 extracts were separated on paper and TLC, assayed by the wheat coleoptile test after which histograms were made (Fig. 3). The data show that growth inhibiting compounds decrease while those which promote coleoptile growth increased with stratification time. The pattern of inhibitor decrease and promoter increase coincides with the improved germination and normal appearance of seedlings from nuts of longer stratification (Fig. 2). The coleoptile growth promotion shown in the histograms was reproducible for any one paper or TLC technique used. However, the agreement between the paper and TLC systems for coleoptile growth promotion was not good. Virtually no growth promotion was exhibited by extracts separated by paper chromatography while it was clearly evident in the middle and lower histograms of Fig. 3 in which extracts were separated by TLC. Though indoleacetic acid (IAA) may be found at an Rf which coincides with the areas of promotion on the TLC histograms, the complete lack of promotion shown on the paper histograms lends considerable doubt as to the nature of the promotion. Tests for cytokinins using *Lemna minor* and gibberellins using the dwarf pea in phase 3 extract were negative.

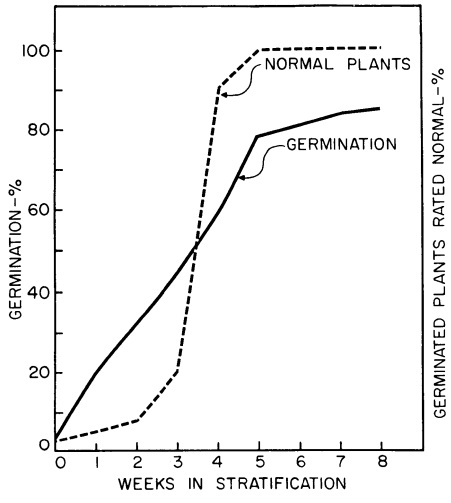

Fig. 2. Cumulative germination in relation to the per cent of those germinated *J. regia* seedlings judged normal after stratification at 0°C.

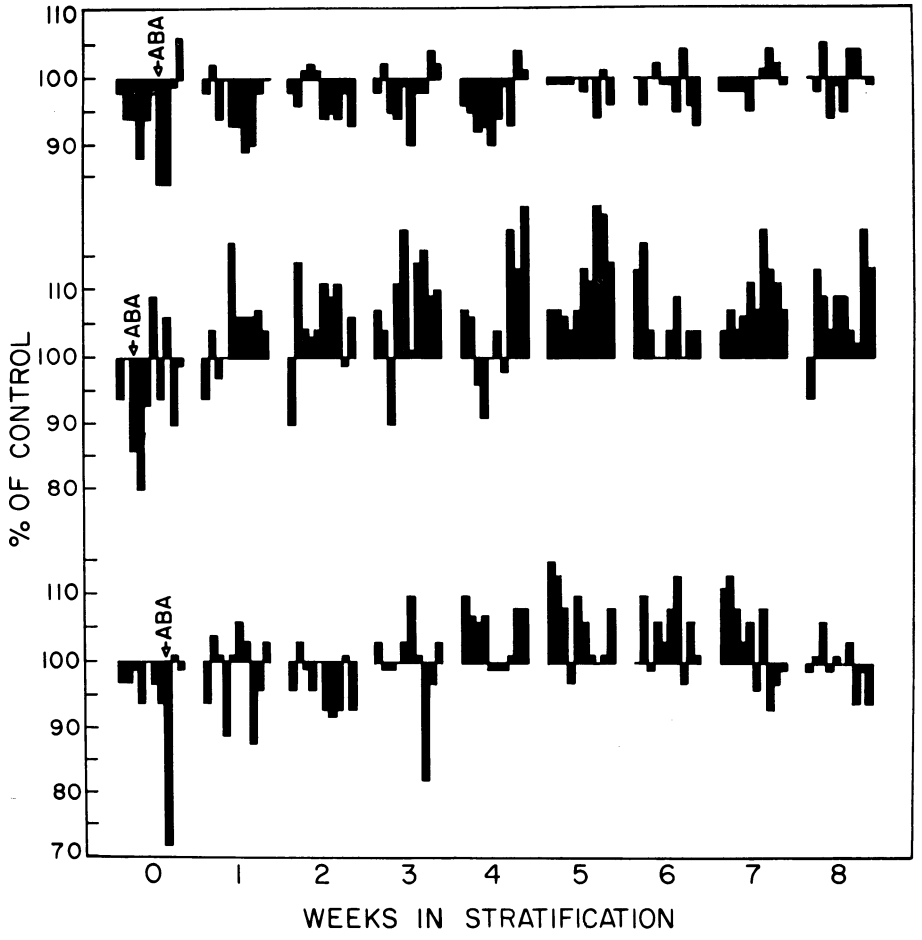

Fig. 3. Histograms of acidic ether-phase 3 showing zones of relative promotion and inhibition during the 8-week stratification period. Upper group developed on 3 MM paper with isopropanol, ammonia and water (8:1:1 v/v/v). Middle group developed on silica gel GF 254 with benzene, methanol and formic acid (85:15:1.3 v/v/v). Lower group developed on silica gel GF 254 with n-butanol, n-propanol, ammonia and water (2:6:1:2 v/v/v). Rf of ABA: upper 0.6–0.7; middle 0.30 and lower 0.70. Rf of IAA: upper 0.35; middle 0.26 and lower 0.55. Extract equivalent to 40, 80 and 40 mg extracted dry weight on top, middle and bottom rows respectively.

Attention was focused on the growth inhibition exhibited by extracts taken from non-stratified kernels (Fig. 3). A relationship was established by co-chromatography between the location of standards of ABA and the inhibition by compound (s) in the adjacent zone. Furthermore, application of ABA showed the equally dramatic inhibition of wheat coleoptile growth which has been substantiated by others (13). The degree of growth inhibition shown by extracts of non-stratified walnuts was equivalent to 0.1 μg ABA.

To elucidate further the nature of the prominent inhibitor, TLC plates were loaded with phase 3 extract taken from non-stratified walnuts developed in BPAW solvent. The zone adjacent to the standard ABA was

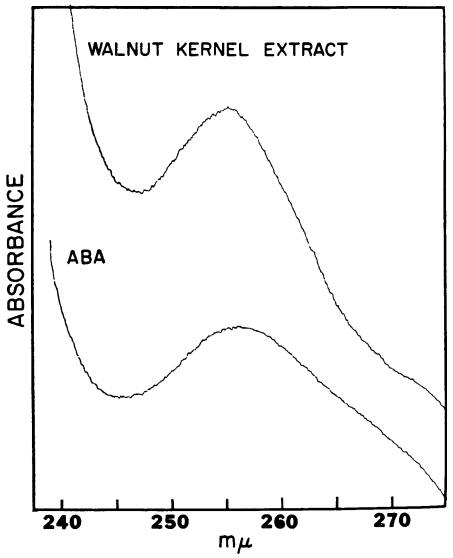

Fig. 4. Comparisons between absorption spectra of inhibitor in walnut kernel and abscisic acid (ABA).

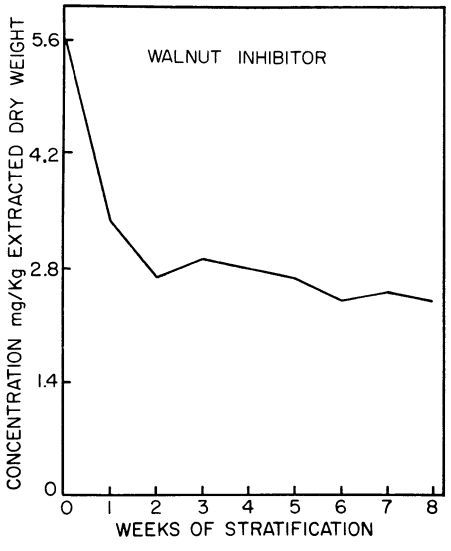

Fig. 5. Relationship between length of stratification and levels of inhibitor in walnut kernels. Abscisic acid was used to establish the standard curve.

scraped from the plate and eluted with 1% acetic acid in methanol. The eluate was concentrated under vacuum and the volume adjusted to 3 ml with 0.005 N H_2SO_4 in 95% ethanol. The spectrum of the unknown was determined with a Beckman DK–2 spectrophotometer and compared to that of ABA. There was good agreement between the spectrums of the 2 materials with the strong absorption maximum at 255 mμ (Fig. 4).

Following the same TLC cleanup procedures described above using either BPAW or BMF solvents another aliquot from the zone adjacent to ABA was reacted with BSA or diazomethane and the derivatives were gas chromatographed. The walnut kernel inhibitor zone developed in BPAW and reacted with BSA resulted in a single peak having the same retention time as a derivative of ABA. On the other hand, when developed in BMF the walnut kernel inhibitor reacted with diazomethane yielded 3 other peaks in addition to one thought to be ABA. Thus the former solvent system, BPAW, appears to have resulted in a better resolution.

An estimation of the quantity of inhibitor in the extracts was made. Following the same TLC cleanup procedures, extracts from walnuts at all stratification periods were assayed using the DU-spectrophotometer. An equivalent of 2 g extracted dry weight in 3 ml 0.005 N H_2SO_4 in 95% ethanol gave readings which were reproducible. The relationship shown in Fig. 5 plotted from spectrophotometer readings clearly demonstrates the dramatic reduction in inhibitor with stratification. The actual values for the inhibitor thought to be ABA were quite high as compared to those published by Milborrow (15). The values varied depending on whether the TLC cleanup solvent was BPAW or BMF. Higher readings were evident with the former solvent.

A final evaluation of the walnut seed inhibitor was made by applying an aliquot to nuts stratified for 8 weeks. Nuts were placed between the jaws of a vice and pressure was applied to ease the shell open at the suture. The amount of inhibitor equivalent to 1.5 g extracted dry weight representing approximately one-half kernel was applied through the opening in the shell to the kernel with a micropipette. Germination of the nuts treated was delayed about 1 week as compared to those which were treated with the carrier solvent only (methanol).

Phase 4—acidic butanol. The presence of gibberellin in the acidic butanol phase, as assayed by the dwarf pea test, could not be detected. No further test was made on the acidic butanol phase.

General. Evidence has accumulated establishing an increase in growth promoting substances in seeds after stratification (14, 17). Also, the application of gibberellin or cytokinin has been shown to enhance germination (11, 16). However, we were not able to detect the presence of gibberellin nor cytokinin in walnut seeds before, during or after stratification. Their participation in breaking dormancy of walnut seeds remains unknown.

Our findings agree with others who have reported the presence of a growth inhibiting compound the level of which is well correlated with the stage of dormancy. Nutile (19) applied coumarin to lettuce seed which induced a dormant condition, while Luckwill (14) and Lipe and Crane (13) working with the apple and peach, respectively, demonstrated the presence of a seed inhibitor which diminished during stratification. Our data disclose that an increase in per cent germination and concurrent growth of normal plants took place as the level of growth inhibitor decreased (Fig. 2, 3, 5). The concentration of the inhibitor reduces sharply the first 2 weeks (Fig. 5) then levels off, while germination increases through 4 weeks before leveling off (Fig. 2). The reason for the lag between the reduction in inhibitor and germination increase is not known, however plant growth responses to internal and external changes are not always rapid. A possible factor might include the rather slow increase in promoters up to 5 weeks stratification (Fig. 3).

The recently identified compound abscisic acid (dormin, abscisin II) has been strongly implicated in the dormancy of seeds and buds (4, 7, 13, 15). Lipe and Crane (13) discussed the reduced level of an inhibitor in peach seeds which occurred during afterripening, and showed that the reapplication of the inhibitor to peach seedlings induced growth cessation and retarded the germination of peach seeds. They tentatively identified the inhibitor as abscisic acid.

The evidence in this report indicates that the level of an inhibitor in the walnut kernel decreases in concentration as the chilling requirement is satisfied. The reapplication of this inhibitor retards germination of walnut seeds that have had their chilling requirements satisfied. The coincidence of the Rf values of the walnut extract inhibitor to that of ABA in several solvent systems (Fig. 3), similarity of the ultraviolet absorption spectra (Fig. 4) the formation of similar derivatives with BSA and diazomethane are circumstantial evidence to suggest the unknown inhibitor to be ABA.

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Effect of Cytokinins and Gibberellins on Shape of 'Delicious' Apple Fruits[1]

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Abstract. The application of cytokinins and gibberellins alone and in combination to 'Delicious' apples just after full bloom affected fruit shape by increasing the length-to-diameter ratio of the fruits. Cytokinins caused fruits to be longer with prominent well-developed calyx lobes. The treated fruit had the appearance of fruits grown where early season temperatures are cool. Gibberellin $A_4 + A_7$ caused fruits to be longer but did not appreciably affect the development of the calyx lobes.

Introduction

SEVERAL factors tend to influence the shape of 'Delicious' apples. Cool temperatures during the cell division and early developmental period cause 'Delicious' fruits to be more elongated, whereas warm temperatures tend to produce round flattened fruits (10). Westwood (12) found that the king or center-bloom fruit was longer and more ideally shaped than the side-bloom fruits, and that crop density and rootstock type influenced 'Delicious' fruit shape.

Chemicals such as gibberellins applied at blossom time affect apple shape by increasing the length-to-diameter (L/D) ratio of the fruit (11, 13). Our purpose was to determine the effect of cytokinins, applied to blossom clusters alone or with gibberellins, on fruit shape.

Materials and Methods

In our experiments several cytokinins, N-(purin-6-yl)-α-phenylglycine (NPG)[3], 6-benzylamino-9-(tetrahydropyran-2-yl)-9H-purine (SD 8339)[4], 6-benzyladenine (SD 4901)[4] and zeatin, were sprayed on 'Delicious' blossom clusters 4 days after full bloom. Experiments were conducted at Wenatchee, Washington, and Hawkes Bay, New Zealand. At Wenatchee two limbs on each of 3 trees were treated with 500 ppm of cytokinin with one treatment consisting of SD 8339 at 500 ppm plus $GA_{4,7}$[5] at 100 ppm and one treatment with $GA_{4,7}$ alone at 100 ppm. At Hawkes Bay 3 limbs on each of 3 trees were treated with 100, 200, and 400 ppm of each cytokinin, with one treatment consisting of SD 8339 at 400 ppm plus $GA_{4,7}$ and one treatment of $GA_{4,7}$ alone at 100 ppm. Each spray solution contained 20% (v/v) ethanol plus .125% Tween 20. At harvest time the length and diameter of 10 of the longest fruits from the control and treated limbs were measured and the L/D ratios calculated.

Results

An effect on fruit shape was evident within a month after treatment. The cytokinin-treated fruits developed more prominent calyx lobes than untreated ones. Data on the effect of the chemicals on fruit shape expressed as L/D ratios are shown in Tables 1 and 2. All of the cytokinins used affected fruit shape, although SD 8339 gave the most consistent results. When both cytokinin and GA_{4+7} were used, very significant fruit elongation occurred. Fig. 1 shows the effect of 3 cytokinins on fruits at 60 days past full bloom. This difference persisted until harvest (Fig. 2) and the treated fruits appeared to be of good quality; firmness tests on the fruit after 5 months in storage at 32°F were essentially the same for both treated and control fruits. An undesirable side effect evident on some fruits was too prominent lobes which gave the fruit a knobby appearance, and a more open calyx. This was especially true of fruits treated in New Zealand. High concentrations (500 ppm) of the cytokinins plus gibberellin accentuated the undesirable effects.

Discussion

The treatments in New Zealand appeared to be more effective than in Washington. This was likely related to the 'Delicious' fruits being rounder and less elongated due to the warmer climate. Under these conditions the potential for affecting fruit shape was

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[2]Crops Research Division.

[3]Crude preparation of NPG made by method of Benes et al. 1965. Nature 206:830.

[4]Supplied by Shell Development Company as experimental compound SD 8339 and SD 4901, respectively.

[5]Supplied by Abbott Laboratories.