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Changes in Indoleacetic Acid, Abscisic Acid, Gibberellins, and Cytokinins during Budbreak in Pecan[1]

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Abstract. **The dynamics of indoleacetic acid (IAA), cis-trans abscisic acid (c,t-ABA), and gibberellin (GA)- and cytokinin-like compounds were measured in pecan [*Carya illinoensis* (Wang) K. Koch; cv. Desirable] buds following their release from imposed dormancy. Both bound and free ABA levels in apical and basal primary buds and in secondary buds declined 27 days prior to budbreak until the time of budbreak, with the exception of a steady rise in free ABA in secondary buds. During this period IAA initially dropped sharply, remained low, and finally increased again at budbreak. Cytokinin-like substances peaked after the drop in IAA but prior to the peak in gibberellin-like substances which occurred during bud swelling. The findings raise the possibility of a key regulatory role for IAA, possibly through its influence on regulation of bud cytokinin and gibberellin levels via their metabolism in roots. Growth regulator dynamics during pecan budbreak are discussed in relation to the hormonal theory versus tissue sensitivity to growth regulators.**

The mechanisms controlling budbreak of pecan have not been elucidated. An understanding of these mechanisms, especially the role of growth regulators, may aid in developing cultural methods that increase nut production by influencing time of budbreak, length of growing season, and the regulation of bud or shoot abortion and consequent pistillate flower production. Evidence from other tree species indicates that fluctuations of growth inhibitors and growth promoters are associated with the control of budbreak (26, 37). Levels of the growth inhibitor, abscisic acid (ABA), declines in buds of *Prunus cerasus* (24), *Betula verrucosa* (12), *Prunus persica* (6), *Fagus sylvatica* and *Ribes nigrum* (39), and *Malus domestica* (30) as dormancy is broken and budbreak progresses. Endogenous levels of growth promoters, such as gibberellins, cytokinins, and possibly auxins, have also been observed to change during the period preceding budbreak. Bioassays for gibberellin-like substances indicate that gibberellins increase between quiescence and budbreak of *Populus balsamifera* (4), *Acer pseudoplatanus* (9), and *Pseudotsuga menziesii* (20). Cytokinin-like substances increased during the same period in *Populus tremula* (10), *Populus balsamifera* (7), *Populus* x *robusta* (13), *Betula paprifera* (7), *Acer saccharinum* (33), and *Prunus americana* (27). Auxin also appears to be important in that auxin content reportedly increases during budbreak in *Pyrus communis* (5).

Most published observations of growth regulator changes leading to budbreak generally have reported on one or rarely 2 hormones. Consequently, comparative levels of bud-located growth regulators is unknown. The purpose of this work was to determine the interrelationship of several growth regulators during the progression of pecan budbreak.

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Materials and Methods

Plant materials and sampling. One-year-old branches were collected randomly during late morning at intermittent periods from early March through early April 1981 from the lower 2/3 of 10 nine-year-old 'Desirable' trees. Samples were bulked for each date, transported to the laboratory, frozen at −20°C, lyophilized, and weighed. Buds were then removed from branches and separated into 3 classes based on their location on the branch: primary buds (1st order auxillary buds) from the apical half of the branch; primary buds from the basal half; and visible secondary buds (2nd order auxillary buds) throughout the full length of the branch. Buds were ground to pass a 40-mesh sieve and stored at −20° awaiting chemical analyses.

Levels of c,t-ABA and IAA were measured by gas–liquid chromatography and levels of gibberellin- and cytokinin-like substances were estimated by bioassay. Free and hydrolyzable (bound) c,t-ABA were measured in all 3 bud classes; IAA and gibberellin- and cytokinin-like substances were measured in primary buds originating from the apical half of the branch. Ten grams of tissue were extracted for determination of each growth regulator at each sample date. All solvents were double-distilled.

ABA extraction and fractionation. The extraction procedure utilized for ABA was a modification of the method used by Takeda and Crane (32). Ten grams of bud tissue were extracted with 100 ml of 80% methanol at 4°C for 24 hr and centrifuged, and the tissue re-extracted with 80% methanol. Extracts were combined and t,t-ABA added as an internal standard.

The t,t-ABA internal standard was prepared by illumination of a methanolic solution of methyl ester c,t-ABA with UV radiation (21); isomeric esters were then hydrolyzed with ethanol-10% KOH (1:1) and separated by thin-layer chromatography (TLC) using chloroform–methanol–acetic acid (97:2:1) (25). Preliminary analysis of a nonspiked bud sample indicated no detectable t,t-ABA.

The spiked extract was evaporated *in vacuo* at 40°C and the aqueous residue adjusted to pH 8 with 1 N NaOH, partitioned with petroleum ether (3 × v), adjusted to pH 3 with HCl, and again partitioned with ethyl acetate (3 × v). The remaining aqueous phase was retained for bound ABA. The acidic ethyl acetate fraction was dried with sodium sulfate and evaporated *in vacuo* at 40°, and the residue dissolved in 0.1 M KH_2PO_4 (pH 8). Contaminating phenolics were removed by shaking the aqueous solution 2 hours with one g of insoluable polyvinylpyrrolidone (PVP) (prewashed with methanol and acetone, then air-dried). After filtering, the filtrate was adjusted to pH 3 (1 N HCl) and partitioned with ethyl acetate (3 × v). The organic fraction was reduced to dryness *in vacuo* at 40° and the residue dissolved in acetone. The acetone solution was then chromatographed with c,t- and t-t,ABA standards using silica gel F-254 TLC plates and toluene–ethyl acetate–acetic acid (40:5:2) as solvent. Standards were visualized by UV light (sample bands were protected) and corresponding zones in the extract immediately eluted using ethyl acetate. The eluate was filtered, and the filtrate dried under N_2 at 40°.

The above aqueous phase containing bound ABA was spiked with t,t-ABA and then adjusted to pH 10 (2 N NaOH) and heated at 60°C for one hour. No attempt was made to measure carryover t,t-ABA from the previous spiking. It was then adjusted to pH 3 (2 N HCl) and extracted with ethyl acetate (3 × v). The organic fraction was then dried *in vacuo* at 40° and the residue prepared for TLC and derivatized as described below.

Gas–liquid chromatography (GLC). The purified samples were dried under N_2 and methylated with diazomethane (29) dissolved in ethyl ether and methanol (9:1 v/v). The derivatives were dried under N_2 and redissolved in dry ethyl acetate for GLC analysis. Levels of c,t-ABA were determined with a Varian 3700 GLC equipped with a ^{63}Ni electron capture detector, and using a 2 mm × 182 cm spiral glass column packed with 3% OV-101 on Chromasorb WP-80/100 mesh. Injection, column, and detector temperatures were 230°, 200°, and 280°C, respectively. Nitrogen flow rate was 30 ml min^{-1}. Percent recovery of t,t-ABA in both free and bound extracts was about 75%. The methyl ester of ABA obtained in the final preparation was identified tentatively by comparison of retention times of the authentic c,t-ABA methyl ester and by its conversion to the t,t-ABA methyl ester upon exposure to UV light (22). c,t-ABA was quantified by measuring peak height and comparing with a standard curve.

Gibberellin extraction and bioassay. The extraction procedure for gibberellin-like substances was similar to that of ABA except that bound levels were not estimated, and no internal standard or TLC step was utilized. The final acidic ethyl acetate fraction was dried *in vacuo* at 40°C. The residue was dissolved in 80% acetone, and the solution passed through a Celite 545-activated charcoal (2:1 w/w) column (25-mm diameter) using 80% acetone (100 ml) as eluant. The eluate was reduced *in vacuo* at 40° and the aqueous residue adjusted to pH 2.5 (1 N HCl) and extracted with ethyl acetate. The organic fraction was then dried *in vacuo* at 40°, flushed with N_2, and stored at −20° awaiting bioassay using the barley ('Himalaya') endosperm method developed by Jones and Varner (18). Their procedure was modified in that the iodide reagent used for the starch reaction was more concentrated (20×) than that reported. GA_3 was used for the standard curve. No estimate was made of percent recovery.

IAA extraction and fractionation. IAA was extracted from primary buds originating from the upper half of the branch using a slight modificaiton of the procedure described by Iino et al. (16) which is designed to minimize IAA decomposition. Briefly, 10 g of bud tissue were extracted in darkness with 100 ml of 80% cold acetone containing 100 mg/l of 2,6-di-tert-butyl-p-cresol (BHT) for 4 hr at 4°C. The mixture was filtered, and the solid residue rewashed with cold acetone. Indolebutyric acid (IBA) was added to the filtrate to serve as an internal standard and the mixture reduced to the aqueous phase *in vacuo* at 35°. Extract pH was adjusted to 8 (2 M K_2HPO_4) and passed through a PVP (3 g) column (25-mm diameter) previously equilibrated with cold 0.1 M K_2HPO_4 (pH 8) and eluted (100 ml) with the same. Elute pH was adjusted to 3 (2.5 N HCl) and partitioned with cold water-washed diethyl ether (3 × 1/2 v) containing 100 mg/liter BHT. The resulting ether phase was then partitioned (3 × 1/2 v) with 50 mM K_2HPO_4 and the aqueous phase adjusted to pH 3 (2.5 N HCl) and partitioned (3 × 1/2) with cold water-washed ether containing 100 mg/liter BHT. The resulting ether phase was dried with Na_2SO_4 and reduced *in vacuo* at 35°. The resulting acidic residue was dissolved in methanol and chromatographed by polyamide TLC with benzene–ethyl acetate–acetic acid (70:25:5 v/v) and 100 mg/liter BHT mixed in the chromatographic solution. Zones corresponding to authentic IAA and IBA were identified by UV visualization (sample zones protected) and scraped from the TLC plates and eluted with water-saturated ethyl acetate. The elute was dried under N_2, and its constituents derivatized for electron capture GLC analysis. Recovery was assumed to be the same as that of IBA (about 70%).

Gas–liquid chromatography. The methyl ester of IAA and IBA were synthesized as described above for ABA. Samples

were then dried under N_2, and methyl esters converted to trifluoroacetyl derivatives by adding 100 µl of trifluoroacetic anhydride and 0.5 mg Na_2SO_4 (14). Unreacted trifluoroacetyl anhydride was removed *in vacuo* and the residue dissolved in anhydrous ethyl acetate for GLC analysis using a ^{63}Ni electron capture detector (14). Measurements were made using 3% OV-101 (Chromosorb W-HP 100/120 mesh support) and tentatively identified by comparing retention times on both 3% OV-101 and 5% DC-200 (Gaschrom-Q 100/120 mesh support). Chromatography was on silanized 2 mm × 182 cm spiral glass columns at 160°C. The detector was operated at 270° and the injector at 200°. Carrier gas (N_2) flow rate was 30 ml min^{-1}. This procedure assumes that methyl-IAA does not occur naturally at significant concentrations in pecan tissue; however, it has been reported in *Citrus* (31). This possibility was evaluated by running samples in which the methyl ester derivization step was deleted before synthesizing the trifuloroacetyl derivative. Since peaks representing methyl-IAA were absent in such samples, natural levels of methyl-IAA appear to be absent or very low in bud tissue.

Cytokinin extraction and bioassay. Primary buds from the apical half of shoots were processed for cytokinin-like substances by a modification of procedures developed by Kannangara et al. (19). Ten grams of bud tissue from each sample date were extracted with 100 ml cold 80% methanol at 4°C for 24 hrs. The mixture was filtered and the solid residue reextracted with another 100 ml of 80% methanol. The combined methanol extracts were reduced to the aqueous phase *in vacuo* at 35° and adjusted to pH 3 (2 M HCl) prior to partitioning against diethyl ether (3 × 1/2 v). The aqueous phase was then adjusted to pH 9.5 (2 M KOH) and repartitioned against n-butanol. The butanol phase was mixed with water and dried *in vacuo* at 40° as the butanol:water azeotrope. The residue was dissolved in 0.05 M KH_2PO_4 buffer (pH 8) and the solution passed through a PVP (2 g) column (25 mm) using the same buffer as eluant. The eluate was adjusted to pH 9.5 (2 M KOH) and partitioned with n-butanol (3 × v), and the butanol phase dried *in vacuo* (as azeotrope). The residue was dissolved in 0.05 M $NaHPO_4$ buffer (pH 7.0) and chromatographed on a Sephadex G-10 column with the same buffer as eluant. The eluate was adjusted to pH 9.5 (2 M KOH) and partitioned against n-butanol, and the butanol phase evaporated *in vacuo*. The residue was dissolved in 80% methanol and bioassayed with cucumber cotyledons according to the method developed by Fletcher and McCullogh (11). However, the procedure was modified in that cucumber seeds (cv. Marketer) were germinated in the dark for 14 days, and cotyledons were excised assay solution for 24 hours rather than for 3 hours. Benzyladenine (BA) was used as a standard. Percent recovery was not determined.

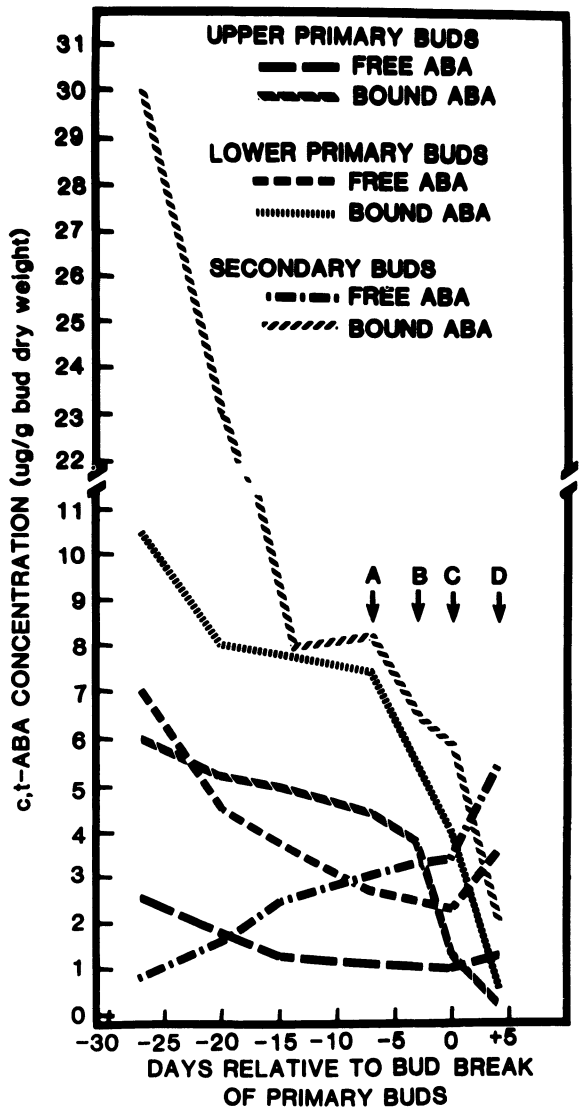

Fig. 1. Concentration changes of free and bound ABA leading to budbreak in buds of 'Desirable' pecan. ABA levels are reported for primary buds originating from the upper one-half and lower one-half of one-year-old branches and for secondary buds throughout the length of the same one-year-old branch. Visual stages of primary bud development are: A) no visual bud swelling or splitting of bud scales; B) bud swelling with splitting of bud scales; C) budbreak (or the appearance of secondary bud scales); and D) bud elongation of 1.5–2 cm with separation of catkin buds from the shoot bud, with both buds still covered by secondary bud scales.

Results and Discussion

Absolute ABA levels were similar to those reported for buds of several other species (12, 24, 39). Levels of free and bound ABA in both classes of primary buds and bound ABA in secondary buds declined with the onset of budbreak (Fig. 1). The decline appeared to be due to a net loss of ABA rather than to dilution during bud enlargement because bud dry weight did not increase until 3 days prior to budbreak and the amount of ABA per upper primary bud steadily declined until just prior to budbreak (Fig. 2). This indicates that both free and bound ABA are apparently being translocated from buds, catabolized, or conjugated in a manner so as to be unextractable with aqueous methanol. Similar ABA patterns are reported in several other tree species (6, 12, 24, 30, 39); thus, ABA appears to be associated with the regulatory mechanism leading to budbreak.

The generally inverse relationship of free ABA levels up until budbreak in both secondary and lower primary buds, coupled with the observation that both bud types broke about the same time, suggests that free ABA *per se* is not regulating budbreak. This suggests that the growth promoter–inhibitor ratio and/or changes in tissue sensitivity is the operative mechanism. The relatively high levels of free ABA in secondary and lower primary buds after budbreak, both of which undergo growth retardation and eventual abscission, suggest ABA is associated with post-budbreak growth inhibition.

The sequence of budbreak for each bud class was upper primary buds developing earlier than lower primary buds and lastly were secondary buds, which correlates very well with levels of

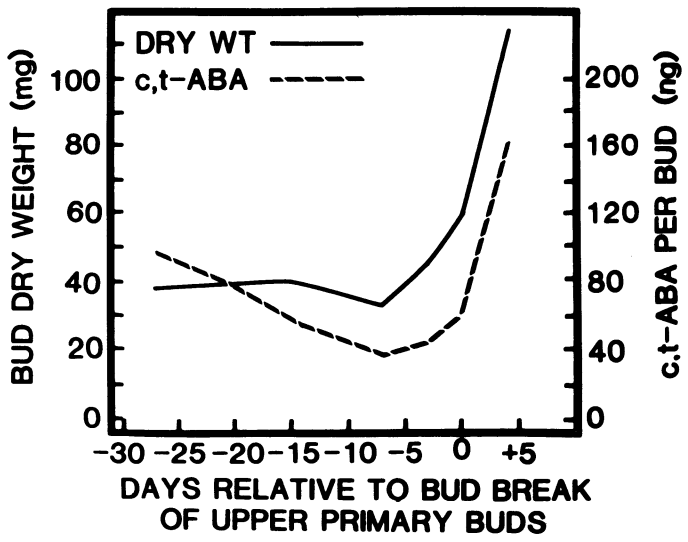

Fig. 2. Dry weight and per-bud abscisic acid (ABA) content of primary pecan buds from the upper one-half of one-year-old branches from quiescence to budbreak.

bound ABA (Fig. 1). This relationship raises the possibility that a portion of the bound ABA is bound to a receptor protein as described by Trewauas (34), and the decline in bud-localized bound ABA parallels a decline in receptor sites. Alcohol-soluable proteins exist in pecan (unpublished observations) and would have been present in the bound ABA aqueous sample fraction. Such a mechanism would indicate that the receptor-ABA conjugates is a regulator of bud development rather than absolute growth regulator levels.

Beginning about one week prior to budbreak, levels of free ABA were lowest in buds that underwent the greatest amount of growth (apical primary buds), greater in buds that grew less (basal primary buds) and eventually aborted, and greatest in secondary buds which grew least of all and were the first to abort (Fig. 1). This suggest that ABA may play a role in correlative inhibition and subsequent abortion of potential nut-producing surface. IAA may also be associated in that previously reported evidence (2) suggests that ABA may be synthesized in lateral buds in response to auxin arriving from apical buds. This possibility is consistent with the sharp increase in IAA levels immediately prior to budbreak (Fig. 3) and may partially explain why all but about 2 buds abort shortly after budbreak. These 2 apical primary buds generally develop into branches and consequently produce pistillate flowers, while remaining buds abort prior to producing visible pistillate flowers. This loss of potential crop and support foliage reduces the production potential of the tree.

Levels of hormones in primary buds from the apical half of one-year-old branches flucuated several fold after the termination of imposed dormancy (Fig. 3). The rapid decline in IAA levels 20–25 days prior to budbreak, subsequent low levels, and eventual sharp rise at budbreak suggest a regulatory role in that IAA may act as a primary signal for growth activation (3). Cytokinin-like substances increased from nondetectable levels to 10 μg g^{-1} dry wt (BA-equivalents) 14 days before bud swelling and declined to relatively low levels at budbreak (Fig. 3). A similar increase in cytokinin activity has been observed in *Populus tremula* (10), *Populus balsamifera,* and *Betula papyrifera* (7). In *Populus* x *robusto* buds (13), zeatin and zeatin riboside were considered to be major components of the cytokinin peak. Since these cytokinins can regulate cell division and differentiation

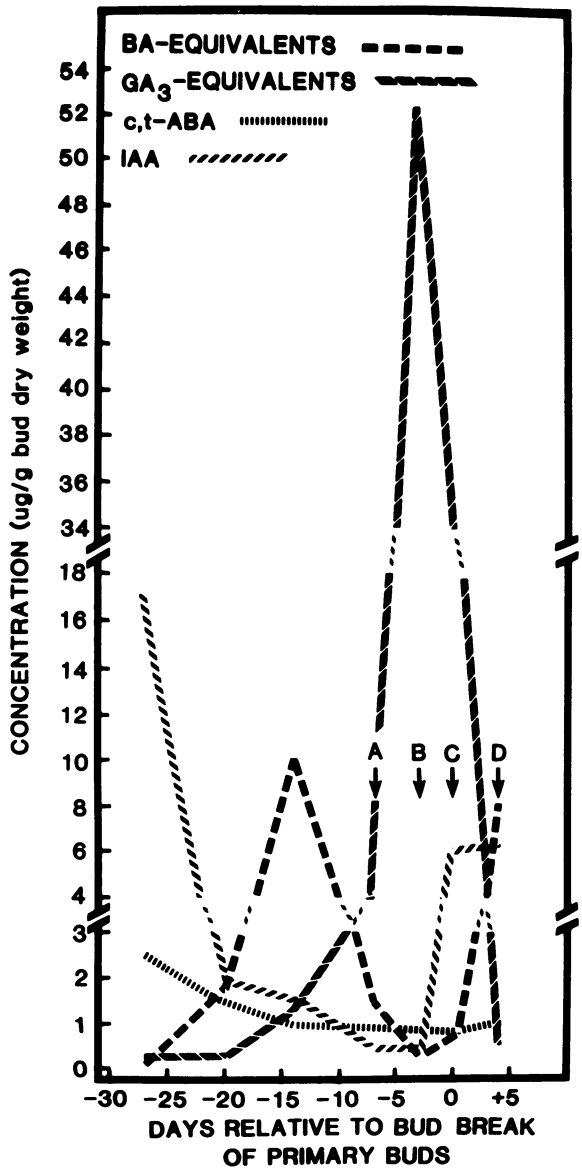

Fig. 3. Concentration changes of free ABA, IAA, and gibberellin-like and cytokinin-like substances leading to budbreak in primary buds from the upper one-half of one-year-old 'Desirable' branches. Visual stages of primary bud development are: A) no visual bud swelling or splitting of bud scales; B) bud swelling with splitting of bud scales; C) budbreak (the appearance of secondary bud scales); and D) bud elongation of 1.5–2 cm with separation of catkin buds from the shoot bud, with both buds still covered by secondary bud scales.

(36), bud growth may be initiated or sustained by cytokinins because ABA, IAA, and detected gibberellin-like compounds are relatively low during this time (Fig. 3). Gibberellin levels also were relatively low 27 days prior to budbreak—a time when ABA and IAA are relatively high. Their maximum occurred about 7–11 days after the peak in cytokinin levels and was associated with bud swelling. Levels were still high at budbreak, but very low immediately thereafter. Similar increases immediately prior to budbreak have been observed in *Populus balsamifera* (4), *Betula pendula* (17), *Acer pseudoplatanus* (9), *Betula pubescens* (35), and *Pseudotsuga menziesii* (20). The above cytokinin–gibberellin pattern indicates that the effects of gibberellins may be partially dependent upon cytokinin levels and may explain why exogenous applications of gibberellins

have been observed to induce budbreak in some plants but not in others (26).

The high levels of IAA and cytokinin and low ABA immediately after budbreak is a natural expectation in actively growing organs; however, the low level of gibberellin is not expected. This may be due to the fact that the barley endosperm bioassay is not sensitive to all gibberellins (28), or because the extraction procedure utilized excluded certain polar (e.g., GA_{32}) and nonpolar (e.g., GA_9) gibberellins that might be active.

Wareing and Saunders (37) postulate that the processes leading to budbreak involve an interaction or balance between endogenous growth promoters and growth inhibitors and is probably the result, rather than the cause, of emergence from dormancy. This view is consistent with the present study in that trees received the required 500 hr (28) of chilling at least 60 days prior to budbreak and the associated events preceeding budbreak were correlated with a decline in IAA and ABA and an increase of cytokinins and gibberellins (Fig. 3).

The pattern of endogenous hormonal changes in pecan buds is similar to that reported by Bachelard and Wightman (4) for *Populus balsamifera*. They proposed an early translocation of auxin to roots from buds in which rest had been broken, resulting in an acceleration of root activity and stimulation of cytokinin and gibberellin production and translocation to buds which accelerates spring growth of buds. This hypothesis appears also to be valid for pecan and is supported by the presence of high levels of IAA in buds 27 days prior to budbreak (Fig. 3) and also prior to the maxima in cytokinin and gibberellin levels. Observations in other species indicate that IAA is basipetally transported from the shoot to root system and can stimulate root activity (36). Rapid growth of pecan roots began in early March (38) when IAA levels in buds were high and also immediately precedes the observed cytokinin peak (Fig. 3). Observations that gibberellin-like compounds can be synthesized in tree roots (20) and that cytokinin-like compounds reach a maximum concentration in *Acer saccharum* with the initiation of lateral root growth with a consequent increase in shoot cytokinin-like compounds (8), plus the observance of cytokinins similar to zeatin and zeatin riboside in the rising sap of *Populus* x *robusto* (13) and transzeatin riboside in the rising sap of *Acer pseudoplatanus* (15), suggest a role for bud-produced IAA in that it may possibly be associated with synthesis and transport of cytokinins and gibberellins from roots to buds. However, literature to support IAA-induced synthesis and transport of cytokinins and gibberellins seems to be lacking; thus, their interrelationship should be investigated.

The accumulation of cytokinins and gibberellins in pecan buds a few days prior to budbreak may, as described, arise by translocation from roots. If so, then reported observations that budbreak occurs in excised branches of pecan following chilling (1, 23) but with consequent failure of continued shoot development (generally attributed to lack of sufficient carbohydrate reserves) is explainable if shoots are excised before adequate accumulation of gibberellins and cytokinins which are translocated from roots (4).

Even though methods used in this study measured mean extractable concentrations of growth regulators in entire buds rather than levels in specific tissues and did not measure tissue sensitivity, pecan budbreak seems to be influenced by the relative balance of growth inhibitors (ABA and possibly IAA) and growth promoters (cytokinins, gibberellins, and possibly IAA). By extrapolation, the data suggest that budbreak of pecan may be accelerated by early exogenous applications of auxins, cytokinins, or gibberellins or inhibited by inhibitors of synthesis or transport of these growth regulators. They also suggest that once buds are broken, their continued development may be induced by the application of growth promoters.

Hormonal concentration changes leading to budbreak varied relatively little (3- to 100-fold), especially in relation to the 5 orders of magnitude exhibited by dose-response curves for most excised and intact tissue systems (34). This relative lack of variability implies that the hormonal theory (development is regulated by altering either absolute or relative concentrations of hormones) does not explain the budbreak mechanism of pecan, but rather tissue sensitivity (abundance of hormone receptor sites changes with development) is the most plausable explanation. Thus, attempts to understand pecan budbreak should also concentrate on tissue sensitivity. Further study is needed of the dynamics of individual cytokinins and gibberellins and the determination of the role of IAA in the early stages of processes initiating budbreak. Monitoring shoot phloem and root exudate for IAA, cytokinins, and gibberellins during the period immediately following quiescence should aid in determining the mechanism of action.

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