HORMONE FLUCTUATIONS DURING STRATIFICATION AND GERMINATION OF BLACK WALNUT SEED[1]

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Abstract.--Seeds collected from each of five black walnut trees were removed from cold stratification at 30 day intervals and dissected into endocarp, cotyledon and embryonic axis. Methanol leachates from each tissue were partitioned against ethyl acetate at pH 2.5 and 8.0. GC (gas-liquid chromatography) and GC-MS (gas-liquid chromatography-mass spectrometry) were used to quantify compounds co-eluting with ABA, GA_3, $GA_{4/7}$, t-zeatin and zeatin riboside.

The highest concentrations of GA's during the first 60 days of stratification were found in the embryonic axis. Abscisic acid concentrations were highest in the embryonic axis, but only during later stages of stratification. Cytokinins appeared to play a permissive role in germination only during the later stages of stratification. Some variation in plant growth regulators in seed from different trees were also observed.

INTRODUCTION

Black walnut (Juglans nigra L.) is one of the most highly prized native hardwoods found in the Central Hardwoods region. Black walnut wood exports represented 4 percent of the total U.S. hardwood volume exported in 1983, but 11 percent of the hardwood export value (McCurdy and Kung, 1985). Thus, black walnut had the highest value of any hardwood species exported averaging $2293.00 per thousand board feet.

The diverse uses of this species and its high value to local and export markets have encouraged the frequent planting of black walnut. Unpredictable seed germination can affect success of direct seeded plantations or supplies of nursery stock. There is a need to obtain a more complete description of factors regulating black walnut seed dormancy to consistently improve germinative capacity.

The seed of Juglans sp. are considered to have a dormant embryo (Brinkman, 1974). Although walnut seeds are morphologically mature, a stratification period (cold, moist storage) is required during which time germination inhibitors decrease or stimulators increase. The suggested stratification period is 90 to 120 days with a 50 percent germinative capacity (Brinkman, 1974).

The occurrence of compounds with the potential to regulate seed dormancy has been investigated for many seeds including the Juglans species. Martin et al. (1969) found no GA's in the kernels of Juglans regia seed, but did identify an ABA-like inhibitor that decreased during cold stratification. Meier (1979) reported the presence of ABA in black walnut seed, but no quantitative measurements during stratification were recorded.

Seed dormancy mechanisms are apparently species specific. The purpose of this research was to characterize, for the first time, the change in hormone concentrations in black walnut seed tissues during the stratification process.

MATERIALS AND METHODS

Mature seed were collected from under 5 trees located throughout Jackson County, Illinois. After mechanically removing the pericarp (fleshy outer portion, or husk), seed from each half-sib family were placed in water to remove floaters. Cleaned seed (200-500) from each family were stored in 4 mil plastic bags inside burlap bags. Seed were cold stratified at 4°C ± 1°C for 180 days, then removed from storage and placed at ambient temperatures for 30 additional days.

Tissue Extraction for Chemical Analysis

At 30 day intervals, 50 sound seed from each family were removed from stratification and opened. If there was no evidence of tissue deteriorati ı, the seed were considered viable. The embryonic axis and some of the cotyledon and endocarp (shell) tissues from 25 sound seed were removed and immediately placed into 80% methanol/water ($MeOH:H_2O$, v:v). A solvent and standards blank, prepared by adding 2 ml of 10 mM stock solution of GA_3, $GA_{4/7}$, c-ABA, IAA, and t-zeatin

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to 25 ml of 80% MeOH, were treated identically to the tissue samples. The tissues were placed on a shaker in a cold (4°C) storage room. After 24 and 48 hours, the tissues were re-extracted with fresh, cold 80% MeOH (100 ml for endocarp and cotyledon tissue, 25 ml for embryonic axis tissue). This process leached free plant growth regulators from the tissues through three changes of 80% MeOH and avoided the usual problem of "cleaning up" complex plant tissue samples which occur when a homogenization step is included (Frankland and Wareing, 1966; Martin et al., 1969; Mann and Jaworski, 1970).

In our experiment, all glassware was acid washed with a solution of concentrated H_2SO_4 and No-Chromix (Godax Laboratories Inc.) for 4-12 hours, rinsed six times with distilled, deionized water, and then dried at 120°C. All containers and vials were capped with aluminum or teflon lined caps.

The methanolic samples were evaporated in a Buchler rotary evaporator at 35° ± 5° C under vacuum to remove the MeOH. The remaining extract was frozen at -80°C and then immediately lypholized. The lypholized extracts were stored at -4°C until all samples were collected. Samples from all tissues, stratification lengths, and families were randomized before hormone analyses were begun.

Hormone Extraction and Analysis

The lypholized cotyledon and endocarp samples were rehydrated with 25 ml of deionized water (Millipore, Milli-Q system), and the embryonic axis samples were rehydrated with 10 ml of deionized water. The rehydrated samples were then partitioned against an equal volume of HPLC grade ethyl acetate at pH 2.5, then pH 8.0 (Figure 1). Both EtOAc fractions were dried under a stream of dry N_2, solubilized in 100 µl of pyridine, and then silyated with 200 µl of BSA (N,O-bis,(trimethylsilyl)acetamide).

Quantitative hormone analysis was performed by injecting 1.0 or 3.0 µl of the silyated sample onto a dual column gas chromatograph (Varian model 3700) equipped with flame ionization detectors. The columns, 180cm x 2.0 mm (id), were packed with OV-1 (methyl silicone) or OV-17 (methyl, phenyl silicone) on 100/120 mesh Gas Chrom Q. Column temperature was programmed from 150°C to 250°C at 8°min^{-1} or 210°C to 280°C at 6°C min^{-1} for acidic and basic samples respectively, using a N_2 flow rate of 40 ml min^{-1}. Duplicate samples of each fraction were run on each column. Chromatographic signals were recorded on a Shimadzu (model CR1-B) integrator/recorder to determine area of peaks co-eluting with hormone standards within a retention time window of 5%.

The internal standards (IS) added to all acidic EtOAc and basic EtOAc samples before drying with N_2 were androsterone (5-alpha-androstan-3 alpha-ol-17-one) and testosterone (17 beta-hydroxyandrost-4-en-3-one), respectively.

The ratio (k_i) of hormone concentrations from the standards sample for the OV17 and OV1 columns was calculated as follows:

$$k_i = \frac{A_i}{B_i}$$

where:

A_i = concentration of hormone i on column OV17
B_i = concentration of hormone i on column OV1

Similar calculations were done for tissue samples. A t-test was used to determine if the k_i ratio of the standard and the tissue samples were significantly different. For each hormone, where the k_i ratio was significantly ($\alpha = 0.05$) greater than the standards samples k_i, the results from the OV1 column were reported (the result suggested more contaminants were co-eluting with the hormone on the OV17 column). Conversely, if the k_i ratio was smaller, the results from the OV17 column were reported. Results from the OV1 column were reported for c-ABA and $GA_{4/7}$ and from the OV17 column for GA_3 and IAA. The mean of the OV1 and OV17 columns were used to report the values for t-zeatin and t-zeatin riboside because there was no significant difference in the k_i ratio for these two plant hormones.

Gas Chromatography-Mass Spectrometry

Standards and plant tissue samples were prepared as outlined above. Mass spectra for eluting compounds were recorded on a Finnigan Model 3000 quadrupole MS equipped with a Finnigan Model 9500 GC using a 150cm (2mm id) column packed with 3% OV17 on 100/120 mesh Gas Chrom Q. Retention times and spectral lines from tissue samples were compared visually to spectral lines obtained from standard samples.

RESULTS AND DISCUSSION

Hormone Quantitative Methods

Percent recoveries of hormones added to standard samples ranged from 27 to 54 percent (Table 1). Recoveries were highest for ABA and the GA's. IAA was not recovered from 75% of the samples because the amount was below the detection limit. The basic fraction had low percent recoveries; however, chromatograms from tissue samples were less complex than those from the acidic fraction so that t-zeatin and t-zeatin riboside could be detected in the tissue samples. The spectral pattern from GC-MS for t-Zeatin from tissue samples matched that

Table 1.--Mean percent recovery of each plant growth regulator added to 28 standard samples.

Standard	Amount Added to Standard (µg/µl)	% Recovery ± Std. Error
Acidic Fraction		
IAA	350/300	28 ± 6
c/t-ABA	530/300	44 ± 4[1]
GA_3	690/300	54 ± 5
$GA_{4/7}$	660/300	51 ± 3
Basic Fraction		
t-Zeatin	440/300	27 ± 4

[1]Calculated as sum of cis and trans isomers. Approximately 6% of cis form was converted to trans form during sample handling.

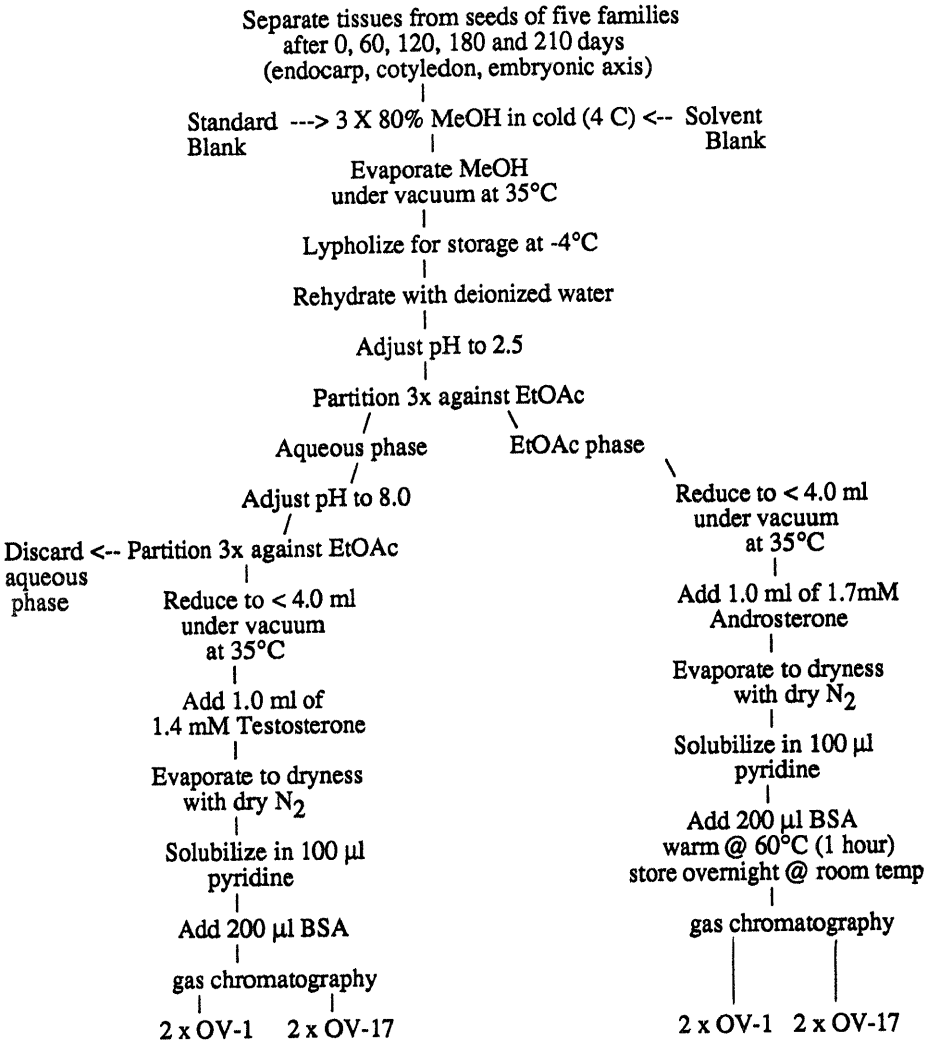


Figure 1.--Flowchart identifying analytical procedures for extraction, purification and gas chromatography of black walnut seed hormones.

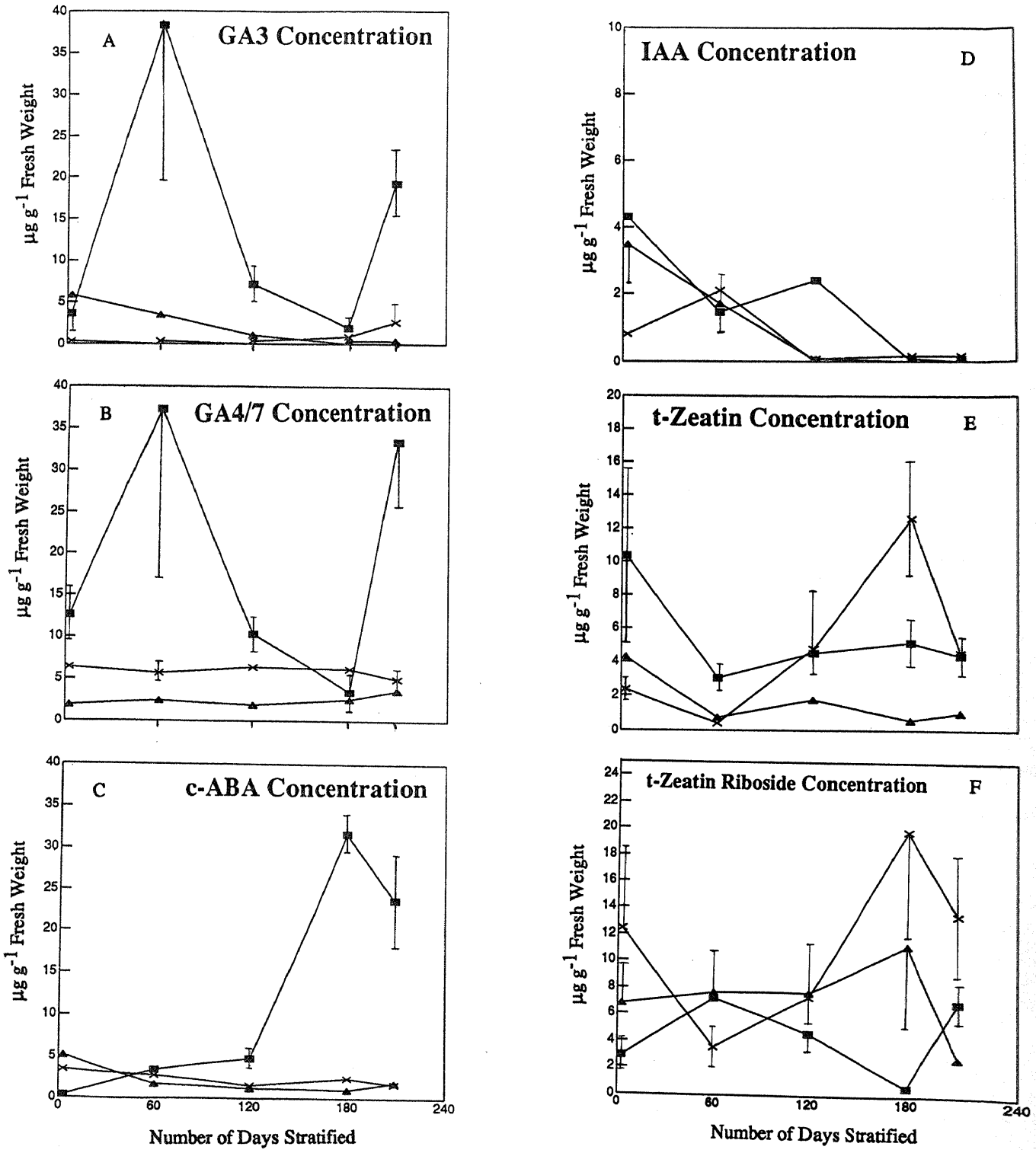
from the standard samples. Because of the number of overlapping chromatographic peaks in the acidic fractions, GC-MS identification of ABA and GA's was not conclusive. The mass spectra for both internal standards (androsterone and testosterone) from the tissue samples matched those from the standard sample.

Percent recoveries reported in the literature range from 10% to 90% for indole-3-acetic acid (IAA) and 59% to 93% for abscisic acid (Brenner, 1979). When quantifying plant growth regulators extracted from natural sources, most of the loss occurs before introducing the sample into the analytical instrument.

Seed Tissue Hormone Levels

The embryonic axis responds to cold stratification during the first 60 days with an increase in compounds eluting at retention times the same as GA_3 and $GA_{4/7}$ (figs. 2A and 2B). After 60 days of stratification, there was a significant decrease in GA's in the embryonic axes. The concentration of GA's again increased in the embryonic axes when seed were removed from cold storage and allowed to begin the germination process (Day 180 to 210).

Black walnut seed dormancy and germination patterns conform well with the model developed by Khan (1975). After seed fall and before cold treatment, some seeds will germinate. Hormone balances resulting from maternal influence are most likely responsible for this phenomenon. However, when the seed are subjected to cold temperatures, none of the seed will germinate until the appropriate endogenous hormone balance has been achieved. Some seeds may germinate after 60 days of stratification, when GA levels have increased in the embryonic axis. During protracted stratification, on the other hand, ABA concentration in the embryonic axis increases and this apparently reduces further growth of the axis. As predicted by Khan's model, an increase in the cytokinin concentration in the cotyledons could have a "permissive" role and allow germination and growth in the presence of elevated ABA levels should environmental factors become favorable.



Figures 2A through 2F - Concentration of plant growth regulators in the embryonic axes (■——■), the cotyledon (×——×), and the endocarp (▲——▲) of black walnut seed during stratification (days 0 to 180) and germination (days 180 to 210). Bars represent the standard error of the mean for points with standard errors larger than the symbol.

In the cotyledons, the GA_3 concentration was below the analytical detection limit until after 120 days of stratification (fig. 2A). However, $GA_{4/7}$ had a constant concentration of 6 $\mu g\ g^{-1}$ fresh weight (FW) throughout the stratification period (fig. 2B). The GA's act as promoters of food reserve hydrolysis in many seeds, this is consistent with ultrastructural studies on black walnut seed which show a continual disappearance of protein and lipid in cells throughout the stratification period (authors unpublished data).

ABA concentration increased in the embryonic axes, but not in the cotyledons (fig. 2C). An increase of ABA late in the dormancy period has been observed in Latuca (Bex, 1972), Sinapis (Schopfer et al., 1979), and Haplopappus (Galli et al., 1980), where ABA may act as an inhibitor of cell expansion in the radicle tissue during late stages of dormancy. After 120 days of stratification, some black walnut seed will rupture the seed coat and begin radicle elongation in the cold; however, the epicotyl usually remains dormant or quiescent until a more favorable temperature is detected.

The role of ABA in black walnut embryonic axes may be to inhibit the growth of the epicotyl during late stages of stratification, when temperatures may not be favorable for epicotyl elongation.

The highest concentrations of IAA occurred before stratification began and generally decreased during stratification (fig. 2D). The decreasing concentration suggests that IAA is probably not involved in the stratification and germination processes within the seed.

There was a rapid increase in t-zeatin and t-zeatin riboside in the cotyledons near the end of stratification (figs. 2E and 2F). The increase in cytokinins in the cotyledons coincided with the rapid increase in ABA in the embryonic axis. Both zeatin and zeatin riboside can substitute for low temperature afterripening in ash embryos (Tzou et al., 1973) and may be acting as "permissive" agents to germination in stratified black walnut seed. Cytokinins have been reported to promote hypocotyl elongation in watermelon seedlings (Loy, 1980) and could enhance black walnut germination after a period of stratification and in the presence of ABA.

Black walnut endocarp tissue showed a decline in all compounds co-eluting with identifiable plant growth regulators during the first 60 days of stratification (figs. 2A to 2F). Concentrations of hormones in this tissue were generally 5 to 10 times lower than in the cotyledons or embryonic axis. Concentrations of hormones in this non-living tissue are most likely a result of residual maternal influences or diffusion from living embryonic tissues.

Although there was insufficient replication to test family differences adequately, we have observed that differences among families may indeed exist. Within a black walnut seed population, there appears to be some half-sib progeny which are more or less responsive to environmental conditions which may break dormancy during a shorter period of stratification. This seems reasonable in the sense that survival may also depend on the ability to remain dormant during a short, favorable environmental cycle; however, progeny which germinate early (after shorter stratification) when climatic conditions continue to be favorable will have a competitive advantage over other species.

CONCLUSIONS

Concentrations of hormones extracted from various seed tissues varied. Concentrations may range over a 1000 fold between tissues of the testa and embryonic axis. For seeds with a dormant embryo that is released after the stratification requirements are met, a promoter:inhibitor hormone relationship has often been found to control dormancy and germination.

Amen (1968) proposed a feedback model of seed dormancy which described the embryonic axis as the site of hormone production which affected enzyme function mainly in the food storage tissues. Similarly, Kelly (1969) reviewed ABA and GA regulation of seed dormancy and germination and concluded that hormones act as the functional components for a cybernetic system which is regulated through the hormonal balance between the embryonic axis and the food storage tissue. This may also be true for dormant black walnut seed, but these models do not fully account for seed which germinate before stratification or during stratification and then remain quiescent until environmental conditions are more favorable.

Probably the most thoroughly tested seed dormancy model is that of Khan (1975). This model compared the possible combinations of three classes of plant growth regulating compounds (GA's, ABA and cytokinins) and their potential effect on seed dormancy. Khan's model assigns a secondary role to inhibitors (e.g. ABA) and is consistent with much of the data reported for dormant and germinating seeds. In addition, this model accounts for germination in the presence of inhibitors (e.g. ABA) by assigning a "permissive" role to cytokinins, which antagonize the effects of inhibitors (Khan, 1971). Khan derived the model by observing that the presence or absence of a hormone or hormones was less important that the relationship between hormones. Khan suggested that there could be many intermediate physiological situations which could cause partial germination or maintain partial dormancy.

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