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Establishment of a callus culture and measurement of seasonal changes in secondary compound production in *Eucommia ulmoides* Oliver

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ESTABLISHMENT OF A CALLUS CULTURE AND MEASUREMENT OF
SEASONAL CHANGES IN SECONDARY COMPOUND PRODUCTION
IN *EUCOMMIA ULMOIDES* OLIVER

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The School of Renewable Natural Resources

by
Erica Deshay Gray
B.S., Louisiana State University, 2000
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ABSTRACT

Eucommia ulmoides Oliver (Eucommiaceae) contains many secondary metabolites, including the lignan pinoresinol di-O- β glucoside (PG) and the iridoid glycoside aucubin (AU), which have been shown to have anti-hypertensive, antioxidant, and hepatoprotective biological properties. The main objective of this research was to create a new agricultural crop in Louisiana for maintaining healthy blood pressure levels. This was done by: 1) determining the seasonal changes in the concentrations of PG and AU in four-year-old *E. ulmoides* trees in cultivation in Baton Rouge, Louisiana using Thin Layer Chromatography and High Pressure Liquid Chromatography (HPLC) and 2) determining production of secondary metabolites of *E. ulmoides* leaf tissue within callus culture using HPLC.

While AU was produced within the cortex and leaves of cultivated trees, PG was not detected. AU was produced year round in both cortex and leaves, except for December and February when leaf abscission occurs. AU was measured in all of the monthly cortex samples, with the April 2001 sample yielding the highest concentration (1.42 $\mu\text{g/g}$). AU concentrations in June, August, and April 2002 leaf samples were not statistically different ($p < 0.05$).

Four levels of NAA (0, 3, 6, 9 mg/l) and BA (0, 1, 2, 4 mg/l) were used to induce callus cells from *E. ulmoides* leaves. AU was not detected by HPLC, but PG was found to be present in 13 of the 16 treatments. A ratio of 3 mg/l NAA to 4 mg/l BA produced the highest PG concentration. The information gained from this data can be used to determine an efficient harvesting scheme for the extraction of secondary metabolites produced by *Eucommia ulmoides*.

CHAPTER 1. INTRODUCTION

1.1 Background on *Eucommia ulmoides*

There are many Chinese medicinal plants, such as ginseng (*Panax ginseng*), *Camptotheca acuminata*, *Ginkgo biloba*, and *Eucommia ulmoides* (Chevallier, 1996, Duke, 1992, and Duke and Ayensu, 1985). Ginseng is valued for its ability to help the body adapt to cold, stress, fatigue, hunger, and temperature extremes (Chevallier, 1996). *Camptotheca acuminata*, a medicinal tree, is the source of two drugs used to treat ovarian and colorectal cancer (Liu et al., 1998). Ginkgo is used as a circulatory stimulant and tonic and also has anti-asthmatic, anti-inflammatory, and antiallergenic properties (Chevallier, 1996). *Eucommia ulmoides* is used in conjunction with many other herbs and has been shown to possess anti-hypertensive effects, along with relieving back pain, frequent urination, and prevention of miscarriage during pregnancy (Chevallier, 1996, Chen et al., 1995, Hu, 1979, and Li et al., 2000). The Chinese have used *E. ulmoides* for thousands of years for its medicinal properties (Hu, 1979). Its name, when translated, means an elm-like tree with hair-like threads. Some of its morphological characteristics are similar enough to that of the Ulmaceae order that *E. ulmoides* was initially thought to be a member of the elm family, as indicated in the name established by Oliver in 1890 (Schnelle, 1990). Eventually, a separate order, Eucommiales, was formed because of some major morphological differences between the elm and *E. ulmoides* (Nemejc, 1956). For instance, the leaves do not have stipules, the flowers do not have perianth, the ovary has two ovules, and the seed has endosperm in *E. ulmoides*. The elm leaves have stipules, the flowers possess a perianth, the ovary has one ovule and the seeds lack an endosperm. *E. ulmoides* is now the sole survivor of its family (Hu, 1979, Tipppo, 1940, and Young and Young, 1992). *E. ulmoides* is also known as the hardy rubber tree, because of the production of gutta percha, a

rubber like resin that can be found throughout the entire plant. The Chinese name for *E. ulmoides* is *tu-chung* or *du-zhong* and is intended to reflect the use of the plant (Hu, 1979).

E. ulmoides is a deciduous, dioecious tree that is native to the central region of China. The bark is a grayish-dark brown color and is smooth when young. As the tree ages, the bark becomes rough with longitudinal lines. It reaches heights of 15 to 20m, with a dense, round, symmetrical crown (Schnelle, 1990). The leaves, at maturity, are dark green with serrated edges. The leaves have an alternate arrangement and reaches lengths of 3.5 to 6.5cm and 2 to 6 cm wide (DeBosier, 2000, and Hu, 1979). The blades are elliptic-ovate, with a rounded base (Chevallier, 1996). During the fall, the leaves do not exhibit a change in color (Schnelle, 1990). The flowers, small, brown, inconspicuous, and without any sepals or petals, bloom in the spring with or just before the young leaves.

The fruit, as described in Magallon-Puebla and Cevallos-Ferriz, (1994) and Hu (1979), is an oblong winged samara, about 3.5 to 4 cm long. The base of the fruit is cuneate, with a notched apex and contains a single oval seed. The fleshy covering of the seed helps it maintain high moisture content. The seed has an oblong shape, is about 14 mm long and has a raphe along the entire length of the seed. The embryo contains a large, stout, white radicle with two cotyledons about 8 mm in length. The embryo is covered in a uniform endosperm. Fruits should be collected in the fall, usually between mid-September and late October, although exact fruiting times vary depending on the region (Dirr and Heuser, 1987, and Hu, 1979).

The natural distribution of *E. ulmoides* is the warm temperate region of China and will grow in US Department of Agriculture zones 5 to 7 (Chevallier, 1996, Hu, 1979, and Schnelle, 1990). It prefers well-drained soils, with neutral pH levels (5.8-8.0), but can also tolerate acidic

soils (pH 5.3-5.8) (DeBosier, 2000, and Hu, 1979). This tree can also withstand periods of drought and has little or no disease or insect problems (Schnelle, 1990).

Because of its medicinal value, *E. ulmoides* is extensively cultivated in China and is rarely found in the wild. The trees are regularly harvested for the bark during the summer and fall. To preserve the tree, only part of the bark is removed, allowing the regeneration of new tissue over the wound (Cui et al., 2000, Hu, 1979, Li et al., 1982, Li et al., 1983; and Zhang et al., 1984). Outside China, *E. ulmoides* is used in urban tree planting, landscaping or in arboreta (Chen et al., 1995 and Hu, 1979).

The bark from large tree trunks are used for medicinal purposes, while bark from smaller trunks, branches, and leaves are used for harvesting gutta-percha (Hu, 1979). Gutta percha, a compound that is found throughout the bark, leaves, and seeds of *E. ulmoides*, has a chemical structure that is similar to natural rubber, explaining the common name, the hardy rubber tree. Gutta percha can be easily seen by breaking or tearing the bark, leaves or seeds into pieces (Hu, 1979 and Schnelle, 1990). It has been used in the production of submarine cables, airplane tires, shoes, and dental material and has electrically insulating properties (Hu, 1979). Gutta percha also has a high resistance to acid, oil and corrosion (Hayman et al., 1994).

Eucommia ulmoides contains many secondary compounds, including lignans, iridoids, terpenes, phenolics, and alkaloids. During the 1980's, thirty-seven compounds found within *E. ulmoides* cortex were isolated and their structures elucidated (Deyama, 1983, Deyama et al., 1985, Deyama et al., 1986a, Deyama et al. 1986b, Deyama et al., 1987). These thirty-seven compounds have a wide variety of biological effects, such as antihypertensive, antioxidant, antimutagenic, antibacterial, laxative, and diuretic (Duke, 1992). The handbook of phytochemical constituents of GRAS (generally recognized as safe) herbs also lists twenty-six

phytochemicals contained in *E. ulmoides*, including aucubin, chlorogenic acid, eucommiol, gutta percha, ulmoside, and hydroxyresinol glucopyranside (Duke, 1992).

While information on *E. ulmoides* in the United States is scarce, the Chinese have comprehensively described the uses of *E. ulmoides*. The flowers and fruit are used as an astrigent, while the entire plant can be used to treat hypertension, neck pains, kidney and liver ailments, and even to prevent hemorrhoids. The bark has an extensive list of uses: analgesic, aphrodisiac, arthritic, diuretic, sedative, impotence, lumbago, rheumatism, back strain, and abortifacient, among others (Duke and Ayensu, 1985).

A three-phase study and perhaps the first of its kind on *E. ulmoides* in the U.S. is being conducted at the LSU Agriculture Center. The goal of this long-term study on *E. ulmoides* is to generate the information needed to develop *E. ulmoides* extracts into an herbal dietary supplement for maintaining a healthy blood pressure level. In this study, leaf and bark samples taken from China were extracted and standardized. The standardized extracts were tested for their safety and efficacy first in animal trials, then in human clinical trials. The first phase, which tested the *E. ulmoides* extracts for their maximally tolerable dose in rats, found that there was no toxic effect from the *E. ulmoides* extracts to the hypertensive rats. The second phase tested the acute toxicity of the *E. ulmoides* extracts, and how efficient the extracts were on lowering blood pressure in hypertensive rats. The third phase, which is currently underway, is human clinical trials. Research from this thesis will become part of this study.

The objectives of this research are to evaluate the *E. ulmoides* saplings that are cultivated at the Burden Research Station in Baton Rouge, Louisiana and to evaluate production of secondary compounds by *E. ulmoides* callus culture. Two experiments were performed to meet these objectives. The first experiment established a callus culture using *E. ulmoides* leaf discs

and petiole sections as explant material. The concentrations of pinoresinol diglucoside and aucubin were measured using high-pressure liquid chromatography (HPLC). The second experiment measured the concentrations of pinoresinol di-O- β glucoside (PG) and aucubin (AU) in *E. ulmoides* saplings by using thin layer chromatography (TLC) and HPLC. Results from these studies will help determine if callus cultures can provide an alternative source of secondary products and what concentrations of these compounds are produced in cultivated *E. ulmoides* saplings at Burden Research Station. This information will be helpful in determining the best time for harvesting cultivated saplings for maximum production of secondary metabolites. Results from this thesis will also test the feasibility of using callus cultures for producing secondary metabolites.

This research will also allow the use of *E. ulmoides* extracts for other medicinal applications. In addition to its use as an anti-hypertensive, the large number of secondary compounds produced by *E. ulmoides* has many uses. AU and other secondary compounds found within *E. ulmoides* have many different biological activities that can lead to new medicines. In addition to harvesting *E. ulmoides* for its anti-hypertensive properties, it could also be harvested to produce new cancer fighting drugs since it has antioxidant and antimutagenic activities. For example, Hsieh and Yen (2000), investigated the antioxidant effects of water extracts of *E. ulmoides* leaves, raw cortex and roasted cortex on oxidative damage in biomolecules. While the leaf extract had an inhibitory effect on breaking DNA strands, the raw and roasted cortex did not have an inhibitory effect at all. Also, in a separate study, an aqueous extract of *E. ulmoides* leaves was found to reduce the frequency of chromosomal aberrations in CHO cells and in mice (Nakamura et al., 1997).

This thesis research resulted in two methods of harvesting PG and AU that are easily applicable to other secondary compounds in the leaves and cortex of *E. ulmoides*. These methods could be used to produce the crude extracts that would be needed in the development and production of new drugs. Application of these results could have a positive effect on Louisiana's economy, hopefully by giving local farmers a Louisiana-based crop and by attracting the highly profitable pharmaceutical industry to this state.

CHAPTER 2. SEASONAL CHANGES IN THE CONCENTRATION OF TWO SECONDARY COMPOUNDS IN *EUCOMMIA ULMOIDES* SAPLINGS

2.1 Literature Review

Eucommia ulmoides Oliver (Eucommiaceae) contains numerous secondary compounds, including lignans and iridoids. Lignans, derived from the shikimic acid pathway, are commonly found in terrestrial plants and have many different biological activities. These include antimicrobial and antitumor activities and also play a role in defensive mechanisms for the plant. Pinoresinol di-O- β glucoside (PG), one of 27 lignans found in *E. ulmoides*, was isolated and synthesized by Sih et al. in 1976 (Figure 2.1). It was also identified as the compound responsible for its antihypertensive properties, and lowers high blood pressure by causing the expansion of blood vessels (Sih et al., 1976, and Hu, 1979). This antihypertensive property has been confirmed in animal studies using aqueous and ethanol *E. ulmoides* extracts. Results of a clinical trial conducted in China demonstrated that 46% of 119 hypertensive human subjects had a significant reduction in blood pressure levels when given *E. ulmoides* extracts orally (Chevallier, 1996). In a separate study, the effects of *E. ulmoides* leaf extracts on 24-hour blood pressure profiles in Japanese subjects were analyzed. Daytime, nighttime and 24-hour blood pressure had a slight but significant decrease within the group that received the *E. ulmoides* leaf extracts, while the group that received a placebo showed a significant increase in 24 hour and daytime blood pressure. These results suggest that *E. ulmoides* leaf extracts have a mild effect in lowering blood pressure (Uezono et al., 1998).

Iridoids are monoterpenoid compounds that are derived from the mevalonic acid pathway. Iridoids are usually found as glucosides and have various biological activities. They are divided into three groups: nonglycosidic iridoid, iridoid glycosides, and secoiridoid glycosides. A single glucose molecule and a closed cyclopentane ring are characteristic of

iridoid glycosides, which include aucubin, geniposide, and geniposidic acid. Figure 2.1 illustrates the similarity in structure of aucubin, geniposide, and geniposidic acid.

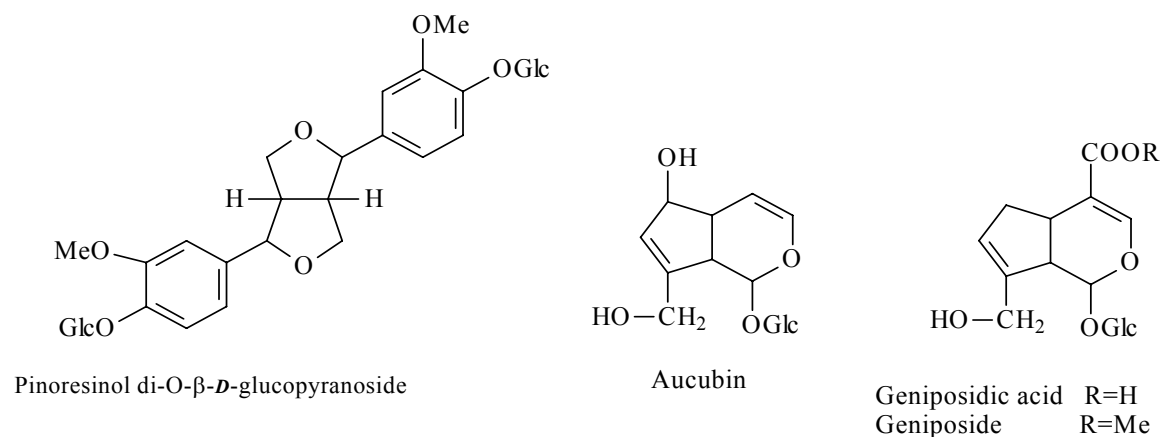


Figure 2.1 Chemical structures of three secondary compounds found within *Eucommia ulmoides*. (Me = methyl group, Glc = glucose)

Aucubin (AU) was first isolated from *Aucuba japonica* leaves in 1902 and has been found in many other plant families, including Apocynaceae, Buddleiaceae, Callitrichaceae, Cornaceae, Eucommiaceae, Globulariaceae, Hippuridaceae, Lentibulariaceae, Loganiaceae, Orobanchaceae, Plantagenaceae, Scrophulariaceae, and Verbenaceae (Battersby and Taylor 1969). Although its concentration is highest in the common plantain (*Plantago major*), *E. ulmoides* is listed as fifth of fourteen plant species with high concentrations of aucubin (Duke, 1992). AU is an iridoid glycoside with a long list of biological activities, most notably, antibacterial, antioxidant, antistaphylococic (Duke, 1992), anti-inflammatory (Recio et al., 1993), liver protectant (Chang et al., 1983; Chang et al., 1984; Lee et al., 2001; Chang, 1998), and collagen synthesis (Li et al., 1998). It is also the antidote for poisonous amanita mushrooms (Chang et al., 1983; Chang and Yamaura, 1993).

Geniposide (GE) and geniposidic acid (GA) also are iridoid glycosides produced by *E. ulmoides*, but these concentrations are relatively low. High concentrations of GE are found in

Euphrasia officinalis L., the fruit and leaves of *Gardenia jasminoides*, and the leaves of *Genipa americana* (Duke, 1992; Endo and Taguchi, 1973; Guarnaccia et al., 1972). Reported biological activities of GE are antibacterial, anticlastogen, antimutagenic, antioxidant, antitumor-promoter, cathartic, choleric, hepatoprotective, antitumor, and laxative (Duke, 1992; Hsu et al., 1997; Kimura et al., 1982; Oshima et al., 1988; Ueda et al., 1991; Wagner and Wolff, 1977; Yamano et al., 1990;). GA is found in high concentrations in the fruit of *Gardenia jasminoides*, the leaves of *Genipa americana*, and in *Plantago major* (Duke, 1992; Guarnaccia et al., 1972). GA has antimutagenic, antioxidant, antitumor, radioprotective and cathartic properties (Duke, 1992; Hsu et al., 1997; Ueda et al., 1991).

These bitter tasting compounds have been shown to have an effect on collagen synthesis. In a study using false-aged rat models, it was shown that GA and AU are among the active compounds that stimulated granuloma formation and collagen synthesis (Li et al., 1998). The effect of aucubin was found to be slightly weaker than the effect of GA. A similar study showed that a water-soluble methanol extract of *E. ulmoides* leaves also led to higher collagen synthesis rates (Li et al., 1999). These studies highlight the fact that the preventive effects on aging are attributed to the secondary compounds present in *Eucommia ulmoides*.

The concentration and production of secondary compounds in *E. ulmoides* are dependent upon many factors. These include harvest time, post-harvest treatment, and production area. Koike et al. (1998) observed the seasonal changes of AU and GA in *E. ulmoides* leaves taken from two separate sites in Japan. Results of the leaves collected from the Funabashi site showed that AU concentrations were up to 2.5% higher in June through September than the rest of the growing season. GA concentrations were 5.5% higher in June and July than the rest of the growing season. The data from the Nakasen site indicated that AU concentrations were 5.5%

higher from July to August, while GA concentrations were 6.3% higher in July than the rest of the year.

In China, AU and chlorogenic acid concentrations were highest in June and November, while GA concentrations were highest in June in *E. ulmoides* leaves (Kangjian et al., 1999a). Flavonoid content was highest in May, and the concentration of gutta percha was highest in May and June. Also, Kangjian et al. (1999b) concluded that ecological factors such as rainfall, temperature, soil acidity, and location all effect secondary compound production. The amount of secondary compounds in *E. ulmoides* leaves varied significantly depending on the regions studied. In addition, Kangjian et al. (2001) determined that genetic factors such as budding, leaf shape, and bark type are important factors in the regulation and synthesis of secondary compounds.

Knowing when a compound of interest is being produced in large amounts is valuable information for the chemical and pharmaceutical industries. The purpose of this study was to measure the concentration of PG and AU in the leaves and cortex of *E. ulmoides* at different times during a complete growing season. This information should help determine the optimal time for harvesting leaves and cortex as sources of the desired secondary compounds.

2.2 Materials and Methods

From April 2001 through April 2002, two branches from each of ten 3-year-old *E. ulmoides* saplings were collected using a pruner. For each tree, the leaves were separated from the branches, put into a paper bag, and allowed to oven dry at 70°C for 72 hours. The dried leaf and cortex samples were ground using a Wiley mill, passed through a 6-mm sieve, weighed and stored in 15 or 50 ml centrifuge tubes at 4°C before extraction.

2.2.1 Extraction

Approximately 100 mg of leaf or cortex samples were weighed and placed into Eppendorf microcentrifuge tubes. For TLC analysis, seventy percent methanol (MeOH) was added to each microcentrifuge tube at eight times the weight (1:8 w/v). For HPLC analysis, the samples were extracted using 70% MeOH for PG analysis and 50% MeOH for aucubin analysis at ten times the weight (1:10 w/v). The samples were then placed on a rotator at a slow speed for at least 72 hours at room temperature. After extraction, the microcentrifuge tubes were centrifuged at 12,000 RPM, 25°C for 10 minutes. The supernatant was removed from the microcentrifuge tube using a needle attached to a 3 ml syringe and was then passed through a 0.2 µm nylon membrane filter into a new microcentrifuge tube. The filtrate was stored at 4°C until used for chemical analysis.

2.2.2 Thin Layer Chromatography (TLC)

Fifteen to twenty microliters of each leaf and cortex sample extract and PG, AU, GE, and GA standard solutions were loaded onto a normal phase TLC plate (Analtech Company, Newark, Delaware, USA) using microcaps and allowed to evaporate.

The mobile phase consisted of 100 ml of 70% chloroform and 30% absolute methanol solution, which was poured into the tank, covered, and allowed to equilibrate under a fume hood for 30 minutes. The plate loaded with the samples was carefully placed into the tank, and then was taken out when the mobile phase was about 1 cm away from the top of the plate. The plate was allowed to dry in a fume hood for 15 minutes, and then placed back into the tank. When the mobile phase was again 1 cm away from the top of the plate, the plate was removed and allowed to dry again for 15 minutes. The developed plate was sprayed with a 15% sulfuric acid solution for visualization of the compounds, and allowed to dry for 15 minutes. Next, the plate was

heated on a stir plate on the lowest heat setting until the samples appeared. The compounds present on the plate, the distance between the spots and the end of the mobile phase, and the R_f values of the four compounds under study were calculated and recorded.

2.2.3 High Pressure Liquid Chromatography (HPLC)

One hundred microliters of each sample was placed into a plastic autosampler vial and capped with aluminum vial seals for HPLC analysis. The filtered samples were stored at 4°C until HPLC analysis. A Beckman System Gold HPLC with a reverse phase C-18 column was used in this analysis at room temperature. The mobile phase in the AU analysis was 9% MeOH in HPLC grade water at a flow rate of 1 ml min⁻¹ for 16 minutes. For PG analysis, the mobile phase was 30% MeOH in HPLC grade water at a flow rate of 1 ml min⁻¹ for 30 minutes. The injection volume was 20 µl, and diode array detection was performed at $\lambda = 205$ nm.

2.2.4 Statistical Analysis

The experiment was a completely randomized design with repeated measures over time. Differences among average AU values were determined by ANOVA using the SAS software. The data was expressed using mean \pm standard error. Differences were considered significant at $p < 0.05$.

2.3 Results

The extracted samples were first analyzed using TLC as a qualitative measurement tool, shown below in Figure 2.1. PG was found only in April '01 leaf and cortex samples, and August cortex samples. (Table 2.1). GA was found in the in the June, August, October, December, February, and April '02 cortex samples, and in the April '01, June, and April '02.leaf samples. GE was present only in the April '02 leaf and cortex samples. AU is indicated to be present in

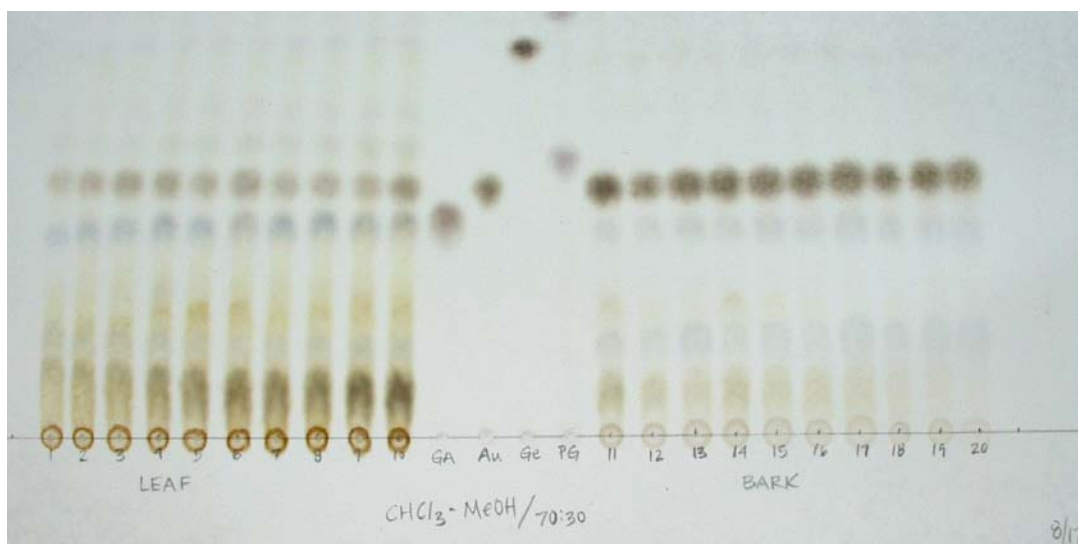


Figure 2.2 TLC plate with April 2001 leaf and cortex samples.

all of the cortex and in all of the leaf samples, with the exception of December and February when the leaves began to dehisce.

Table 2.1 Presence (+) or absence (-) of pinoresinol diglucoside (PG), geniposidic acid (GA), geniposide (GE), and aucubin (AU) in the leaf and cortex samples of *Eucommia ulmoides*, as determined by TLC. *Leaves were not available to collect due to season.

	PG		GA		GE		AU	
Month	Leaf	Cortex	Leaf	Cortex	Leaf	Cortex	Leaf	Cortex
April '01	+	+	+	-	-	-	+	+
June	-	-	+	+	-	-	+	+
August	-	+	-	+	-	-	+	+
October	-	-	-	+	-	-	+	+
December	-	-	-	+	-	-	*	+
February	-	-	-	+	-	-	*	+
April '02	-	-	+	+	+	+	+	+

The concentrations of secondary compounds observed by TLC were quantified by HPLC using standard calibration curves. The secondary compounds that were indicated to be present from the TLC analyses were validated by comparing the retention time and UV spectrum of the peaks of the compounds present in the samples to those of standards. PG was not detected in any of the monthly leaf or cortex samples using PG's characteristic UV absorption spectrum from

190nm to 440nm. AU's presence was confirmed and quantified in both the leaf and cortex samples by HPLC. The month and type of sample were both found to have a significant effect on aucubin concentration (Table 2.2)

Table 2.2 ANOVA for the effects of season and sample type on aucubin (AU) production in two-year-old *Eucommia ulmoides* saplings.

Effect	Num DF	Den DF	F value	Pr>F
Month (cortex)	6	54	34.90	<.0001
Month (leaf)	4	32	3.45	0.0187

The cortex samples produced AU in every monthly sample, while the leaves were found to contain AU only in June, August, and April '02. The April 2001 cortex sample has the highest mean aucubin concentration (Figure 2.2) and is significantly different from the August, October, December, February and April '02 cortex samples ($p < 0.0001$). The April and June cortex samples are not significantly different from each other.

Figure 2.3 shows that there is no significant difference in aucubin concentrations between leaf samples, although the ANOVA table indicates a significant difference ($p=0.0187$). This is because no pairwise difference between the leaf samples was detected. Aucubin is found in the June, August, and April '02 leaf samples, but was not found in the April '01 and October leaf samples. Figure 2.3 also illustrates the accumulation of aucubin within the leaf and cortex samples. From this figure, it is apparent that aucubin production in the cortex samples peaked in April 2001, decreased to its lowest concentration in October, then increased in December and February, and finally decreased in April 2002. The leaf samples appear to produce aucubin during the spring and summer, with no production during the fall and winter months.

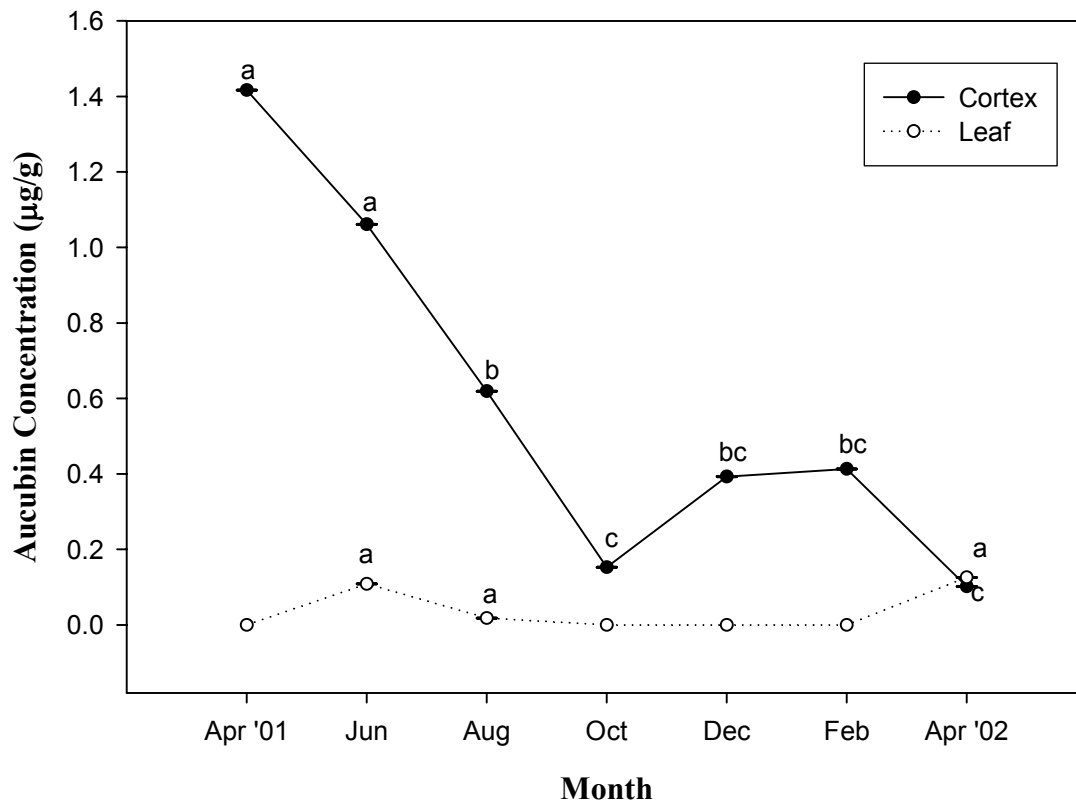


Figure 2.3 Average aucubin concentration (µg/g) in *Eucommia ulmoides* cortex and leaves. December and February did not produce leaves. Means with different letters among sample types are significantly different at $p \leq 0.05$. Vertical bars at each data point indicate one standard error of the means.

Rainfall totals recorded at Burden Research Station and average monthly temperatures in Louisiana statistics were taken from the Louisiana Office of State Climatology monthly newsletters. In 2001, total rainfall levels at Burden Research Station were near normal levels during January and February (Figure 2.4). March rainfall totals were more than twice the levels that fell during the previous two months. April 2001 saw a sharp reduction in rainfall totals, with only 0.77 inches reported. In June 2001, a tropical storm caused over 20 inches of rain to fall. The remainder of 2001 continued to have significant rainfall levels, with the average annual rainfall levels being ten inches above normal. The rainfall totals for the following year exhibited a similar trend during the months of January, February, and March. Although April 2002 rainfall

totals did not decrease as sharply as it had in 2002, it was still considered to be a relatively dry month.

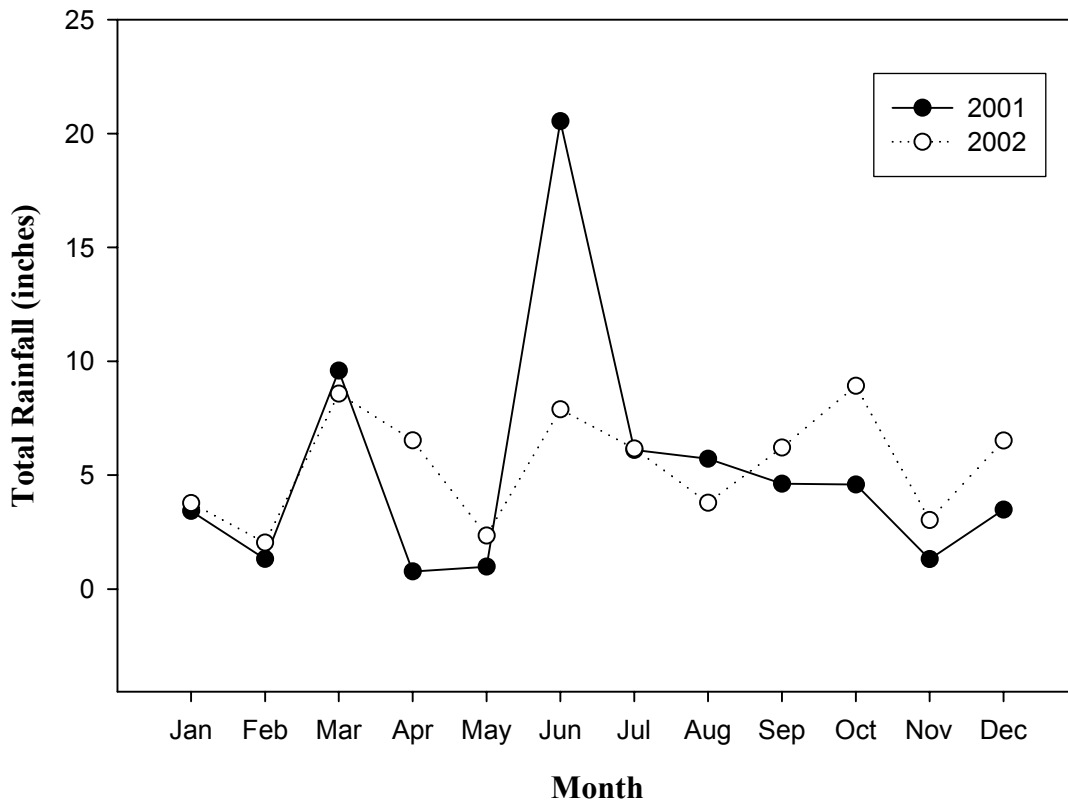


Figure 2.4 Total monthly rainfall (inches) at Burden Research Station in Baton Rouge, Louisiana for 2001 and 2002. Data taken from the Louisiana Office of State Climatology (LOSC), Department of Geography and Anthropology, LSU, Baton Rouge, LA.

Figure 2.5 presents average monthly temperatures in Louisiana during 2001 and 2002. For both years, January, February, and March maintained average temperatures that were lower than 15.6 °C. Although the February 2001 statewide average temperature was 5.2 degrees above normal, it did not set record warmth levels. The February 2002 average temperature was only slightly below normal, and ended a three-month streak of warm weather. From April on to June, average temperatures steadily increase to about 26.7° C, then reaches a plateau in July and

August. September through December shows a steady decrease in temperatures, with December averaging 10-12.8 °C.

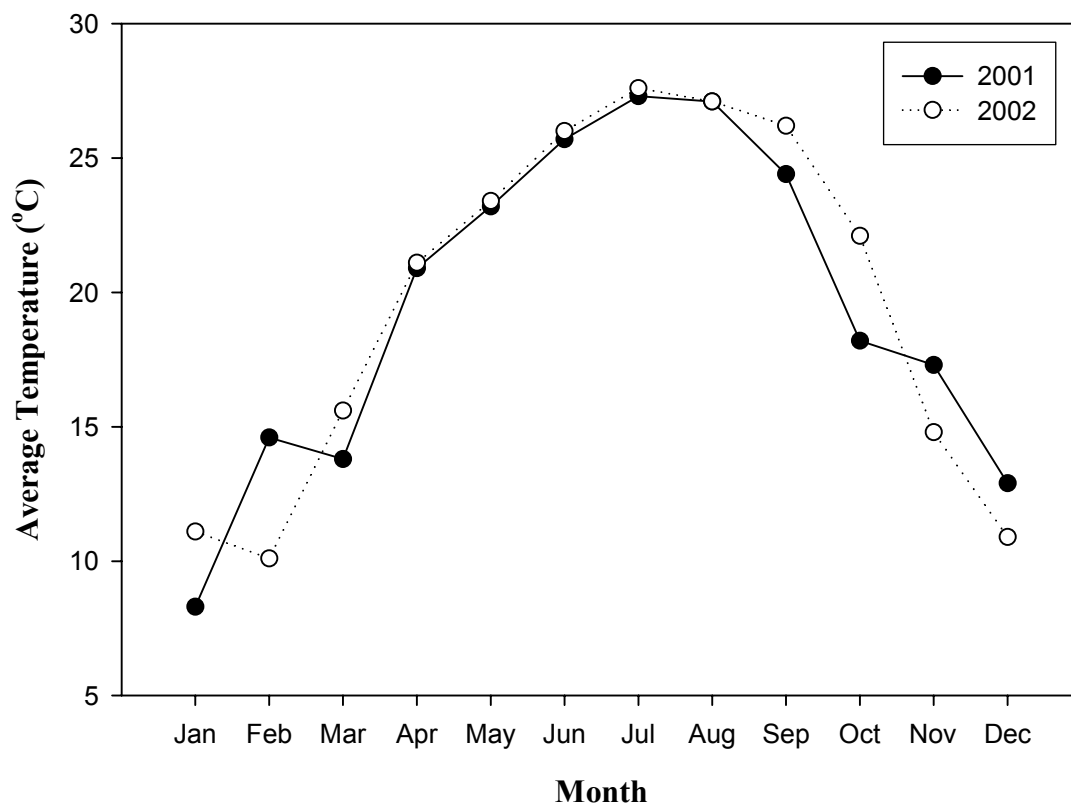


Figure 2.5 Average monthly temperatures in Louisiana (2001-2002). Data taken from the Louisiana Office of State Climatology (LOSC), Department of Geography and Anthropology, LSU, Baton Rouge, LA.

2.4 Discussion

Very few experiments on the seasonal changes of secondary metabolites in *Eucommia ulmoides* have been performed, and to the author's knowledge, no such experimentation has been performed in the United States. Previous investigations indicate that there are significant changes in the concentrations of secondary metabolites over time (Kangjian et al., 1999a, Kangjian et al., 1999b, Kangjian et al., 2001 and Koike et al., 1998). In Japan, it was found that AU concentration in *E. ulmoides* leaves were up to 2.5% higher from June to September at one

site and 5.5% higher from July to August at another site (Koike et al., 1998), while a Chinese study reported high AU levels in *E. ulmoides* leaves in June and November (Kangjian et al., 1999a). These previous findings do not correspond with the present data gathered which shows that there is no significant difference within a growing season that produced AU in *E. ulmoides* leaves. However, it was found that AU was produced in the cortex in every month sampled, with the highest concentrations found in April and June (Figure 2.3). Finding AU in the cortex samples corresponds with other reports on the constituents of *E. ulmoides* cortex (Deyama et al., 1986; Deyama, 2001; Deyama et al., 2001; and Oshima et al., 1988). While the literature states the presence of AU within *E. ulmoides* cortex, there are no other studies that have been performed outside the United States that resulted in similar AU variation patterns within a growing season. This is the first report on the patterns of AU on *E. ulmoides* trees cultivated within the United States.

There is an unexpected difference in AU concentration between April '01 and April '02 leaf and cortex samples. The April 2001 leaf samples did not produce AU, while the following April leaf samples did produce aucubin, and the April 2001 cortex samples produced thirteen times the amount of AU than the amount produced in April 2002 (Figure 2.3). Many biological and ecological factors, such as rainfall and temperature, could have played a role in attributing to the lack of AU in the leaves in the second April. These factors, among others, are known to have an effect on secondary metabolite synthesis (Kangjian et.al, 1999b). For instance, there is a difference in rainfall totals between April 2001 and April 2002 (Figure 2.4). For both years, rainfall totals in March were above normal. In April 2001, there was a sharp decrease in total rainfall, while in April 2002, the total rainfall level was near normal levels. This difference in rainfall totals can have an effect on soil conditions, and also AU production. During that time

period, the soil was probably more saturated in April 2001 than it was in April 2002. It is possible that the stressful conditions caused the decrease in AU production in April 2002 (Figure 2.4). Although the statewide average temperatures for April 2001 and April 2002 were slightly above normal, it may not be a factor in the decrease in AU concentration in April 2002 since the averages were not significantly different. The lack of consistency in the presence of a secondary compound over time indicates that the biosynthesis of aucubin does not follow a chronological clock. In fact, the presence or absence of a particular secondary compound is an expression of comprehensive metabolic reactions taking place in the plant. For example, tree age played a role in the accumulation of camptothecin in the leaves of *Camptotheca acuminata*, with aged trees showing a much reduced concentration in the same chronological month but in different years (Liu et al. 1998).

Since PG has been found in cortex in Chinese experiments, it was thought that PG would be produced in the United States as well. There are mixed reports on the presence of PG in leaves of *E. ulmoides*. PG is listed as the active compound an anti-hypertensive agent in *E. ulmoides* cortex, but its presence or absence in *E. ulmoides* leaves is not discussed in Deyama et al., 2001, Hu, 1979 or Sih et al., 1976. However, there are reports that indicate the presence of PG in the leaves. Deyama et al. (2001) states that leaves have been used instead of cortex for its anti-hypertensive properties since the 1970's. Since the leaves and cortex both contain common iridoid and lignan compounds, it is easier and more profitable to use the leaves for harvesting than to use the cortex.

Finding that PG was absent from both the cortex and leaf samples in this study was not expected, since the bark can contain, an average of 0.1% to 0.5% PG (Deyama et. al 2001). One possible reason for this absence may have to do with the tree age when the bark is harvested for

medicinal use. In China, all the bark is harvested when the trees are mature (Deyama et al., 2001; Du, 1996). This harvesting scheme is based more on the yield of the bark production than the content of PG. This study finds that the bark of three-year-old plants grown in southern Louisiana contains an undetectable concentration of PG. Although South Louisiana is similar enough to *E. ulmoides*'s natural distribution area, there is one difference that may have a significant effect on secondary metabolite production. *E. ulmoides* thrives in a climate with sharp seasonal changes with average temperatures of 0-2°C in January, and 24-28°C in July. In China, there is a definite change in seasons and a well-defined winter in the area where *E. ulmoides* grows, something that is somewhat ambiguous and short-lived in South Louisiana. It may also be too warm during the summer in South Louisiana, since scorched leaves were observed during the August collection. These ecological factors are known to have an effect on secondary compound production and may be an explanation for the lack of PG in the South Louisiana saplings. As trees age PG may be synthesized or accumulated in the bark in high enough concentrations to be detected, a hypothesis worthy of further investigation.

This information is valuable since there has not been any investigation on secondary metabolite production in *E. ulmoides* grown in the United States. It enables the determination of a harvesting scheme for *E. ulmoides* sources in the United States, rather than relying on information gathered from Japan and China. These data indicate the best time to harvest *E. ulmoides* leaves for AU is between April and August and the best time to harvest *E. ulmoides* cortex is between April and June. Since AU is regularly produced in the cortex, and is produced in larger concentrations in the cortex than in the leaves, the cortex may be better suited for harvesting. However, harvesting leaves presents a sustainable scheme that allows yearly harvesting activities, as compared to the cortex harvesting that is much less frequent within a

growing season. Choosing which scheme is best will largely depend on the amount of time and money provided.

The quantification of AU by HPLC and detection of secondary metabolites by TLC in this thesis research is important also for the pharmaceutical industry as well as Louisiana's economy. *E. ulmoides* is a tree with many phytochemicals that are waiting to be transformed into a new healthcare product. Its effect on lowering high blood pressure is very promising, and considering how widely spread hypertension is in the general population, a new form of treatment that does not have the side effects linked to the medications presently in use would be a better complementary and alternative medicine.

CHAPTER 3. CALLUS INDUCTION OF *EUCOMMONIA ULMOIDES*

3.1 Literature Review

Tissue culture of plant tissues is a method that has a wide variety of applications, including micropropagation, embryogenesis, organogenesis, and callus culture. The practice of tissue culture is used by different industries, such as the horticultural, agricultural, and pharmaceutical industries. Since plant cells are totipotent, using cells to produce new plantlets or its products can shorten the time needed by eliminating the need to wait until a plant or tree matures. Callus and suspension cultures have been used to produce secondary products that have medicinal properties or other uses such as natural flavors or dyes in a sterile environment. The induction of callus, which is a cluster of undifferentiated cells, begins with a small section of plant tissue or explant that is manipulated using plant growth regulators to induce the production of calli. Usually, an equal ratio of auxins and cytokinins will give the desired effect, but each species will require its own specific combination of concentrations.

Previous experiments have shown that callus cells can produce the same secondary compounds that are produced in whole plants, but the concentrations are often different (Brown and Charlwood, 1986). This is because the callus cells produced from the explant material are usually heterogeneous, and will contain more than one cell type. A study by Nakawaza and Toda (1995) showed that the amount of the secondary metabolites PG, GA, and syringaresinol di-O- β -D-glucopyranoside produced in *E. ulmoides* were half the amount produced in bark and leaves. Chlorogenic acid is another secondary metabolite that can be produced by callus or suspension culture. Wang et al. (2003) used capillary electrophoresis to determine the chlorogenic acid content of suspension cells from *E. ulmoides* cortex. The results showed that the mean content was 2.15%, which is similar to the chlorogenic acid content found within *E.*

ulmoides leaves. In a related experiment, Zhang et al. (1997) used reverse electroosmotic flow capillary electrophoresis to separate and determine the concentration of chlorogenic acid in *E. ulmoides* leaf and cortex cell culture, and developed a method of determining chlorogenic acid content that can be completed within four minutes. Tang et al. (2002) induced callus using Harada medium supplemented with 0.5 mg/l 2,4-D and 0.6 mg/l BA and in Bourgin and Nistch supplemented with 0.5 mg/l 2,4-D and 0.6 mg/l BA. Results showed that the growth rate of callus and production of chlorogenic acid was significantly influenced by culture conditions such as temperature, carbon source, medium type and plant growth regulator concentration and that the conditions that are favorable for callus growth are different from those required for chlorogenic acid accumulation in callus tissues. These results indicate a need for a more refined method or protocol that defines which media type and PGR is best for maintaining *E. ulmoides* callus cultures for harvesting natural products.

In other experiments that used *E. ulmoides* as explant material, callus cells have been initiated using different types of media, including MS (Murashige and Skoog, 1962), Gamborg's B5 (Gamborg et al., 1968), Lloyd and McCown's Woody Plant Medium (WPM) (Lloyd and McCown, 1980) and a wide variety of auxins and cytokinins. Several studies focused only on the induction of callus tissue and how to optimize callus growth. For example, Tang et al. (1997) found that the callus induction rate of leaves from a coarse-bark cultivar is significantly higher than the rate from a smooth-bark cultivar. Also, the callus induction rate of explants taken from 10-15 day old leaves was significantly greater than that of 20 to 25 day old leaves, while leaves older than 30 days did not produce callus.

Wang et al. (1997) first induced callus using MS medium, then suspended the calli in liquid MS medium. The results showed that using the cytokinin 6-benzyladenine (BA) alone

produced tight, green calli that could not be used in suspension culture, while using BA and 2, 4-D together produced loose, light yellow callus that gradually became tight and brown. Using only 2, 4-D resulted in loose calli suitable for suspension culture.

Zhu et al. (1997) used endosperm from *E. ulmoides* seeds to induce callus in hopes of establishing a new triploid type. After 40-50 days in culture, a soft callus was formed that needed to be subcultured before they would redifferentiate and multiply. Although shoot redifferentiation was achieved 2-5% of the time using 1.2 mg/l BA and 0.15 mg/l NAA, most were abnormal shoots. Normal shoot redifferentiation occurred at a rate of 0.1-0.01%. About 2,000 plantlets were propagated from the normal regenerated shoots and from these plantlets, 12 successfully survived root induction and transferal to natural conditions. This regeneration from endosperm culture was the first reported for *E. ulmoides*.

A mathematical model for the analysis of the mass transfer of nutrients across *E. ulmoides* callus cultures was proposed by Xing et al. (2001). This model would identify the limiting factor responsible for callus growth and enlargement during culture. The information gained from this model would then allow the mass production of a factor enhancing collagen synthesis in animal cells. These studies show that *E. ulmoides* is able to sufficiently produce callus under a variety of conditions, but with some differences in PGR ratio and other conditions to achieve the desired product, which can range from plant regeneration to alternative methods for producing secondary compounds.

3.2 Materials and Methods

3.2.1 Media Preparation

The medium used for callus culture was Woody Plant Medium (WPM). The WPM medium (Sigma Chemical company, St. Louis, Missouri) contained 4 g/L WPM basal salts, 30

g/L sucrose, and 1.5 g/L Gelrite®(Chen et al., 1995, DeBosier, 2000). There were 16 treatments in this experiment, with factorial combinations of four levels of NAA (0, 3, 6, 9 mg/L) and four levels of BA (0, 1, 2, 4 mg/L). Each treatment was replicated ten times.

3.2.2 Sterilization of Explant Material

Young leaves from three-year-old *E. ulmoides* saplings located at Burden Research Station, Baton Rouge, and L.A. were used as explant material. The samples were taken in August 2001. The leaves were disinfected by first rinsing in sterilized distilled deionized water (DDH₂O) for 15 minutes. Next, the leaves were rinsed in a 10% bleach solution (1% sodium hypochlorite) with two drops of Tween 20 for ten minutes, and then rinsed three times at five minutes each with sterile DDH₂O. The leaves were allowed to soak in a 70% ethanol solution, with two drops of Tween 20 for three minutes, and then rinsed with sterile DDH₂O three times for five minutes each rinse. Finally, the leaves were soaked in a 1% bleach solution with two drops of Tween 20 for ten minutes, followed by rinsing with sterile DDH₂O three times for five minutes each rinse.

3.2.3 Inoculation of Explant Material

Under a laminar flow hood, an autoclaved paper hole punch was used to cut out leaf discs of uniform size and shape. The leaf discs were placed adaxial side down into 50 ml test tubes containing 15 ml of WPM media. Leaf petioles were cut using a scalpel and placed into 50 ml test tubes containing 15 ml of WPM media. The explants were then placed in an incubator at 25°C under a 16-hour photoperiod, and were allowed to incubate over a twelve-month period. The resulting callus tissue was transferred to fresh media every three months.

3.2.4 Extraction

At the end of the incubation period, the tissues were placed into 15 ml centrifuge tubes and freeze dried. The dry weight was determined, and the samples were extracted using 60% methanol. The samples were shaken for 72 hours, and then centrifuged for 15 minutes at 10,000 rpm on a Beckman centrifuge. The liquid supernatant was drawn off using a needle and a three ml syringe, and then passed through a 0.2 μm nylon or cellulose filter. The filtered samples were concentrated until dry or nearly dry using a nitrogen blower to blow air into the samples, and then reconstituted to a volume of 500 μl using 60% methanol. Finally, 200 μl of the samples were placed into labeled autosampler vials, and then covered with aluminum caps.

3.2.5 HPLC Analyses

The samples were analyzed for the presence of PG and AU using a Beckman System Gold HPLC with a reverse phase C-18 column at room temperature. The mobile phase used for the aucubin detection was 9% MeOH in HPLC grade water at a flow rate of 1 ml min⁻¹ for 16 minutes. For PG analysis, the mobile phase used was 30% MeOH in HPLC grade water at a flow rate of 1 ml min⁻¹ for 30 minutes. For both analyses, the injection volume was 20 μl , and diode array detection was performed at $\lambda = 205$ nm. The data was analyzed using ANOVA to determine significant differences between sample concentrations and a Tukey's test was performed to determine mean separation.

3.3 Results

The explant materials were not labeled during the last subculture, making it impossible to determine the difference between calli formed from leaf discs of leaf petioles. All of the treatments, with the exception of the control, resulted in the production of callus cells. Figure 3.1 illustrates the dry weights of the calli formed in this experiment. Treatment ten, which was a

ratio of 6 mg/l NAA to 1 mg/l BA, produced the highest dry weight concentration. This treatment is significantly different from treatments 1, 2, 3, 4, 5, 8, and 14(Figure 3.1).

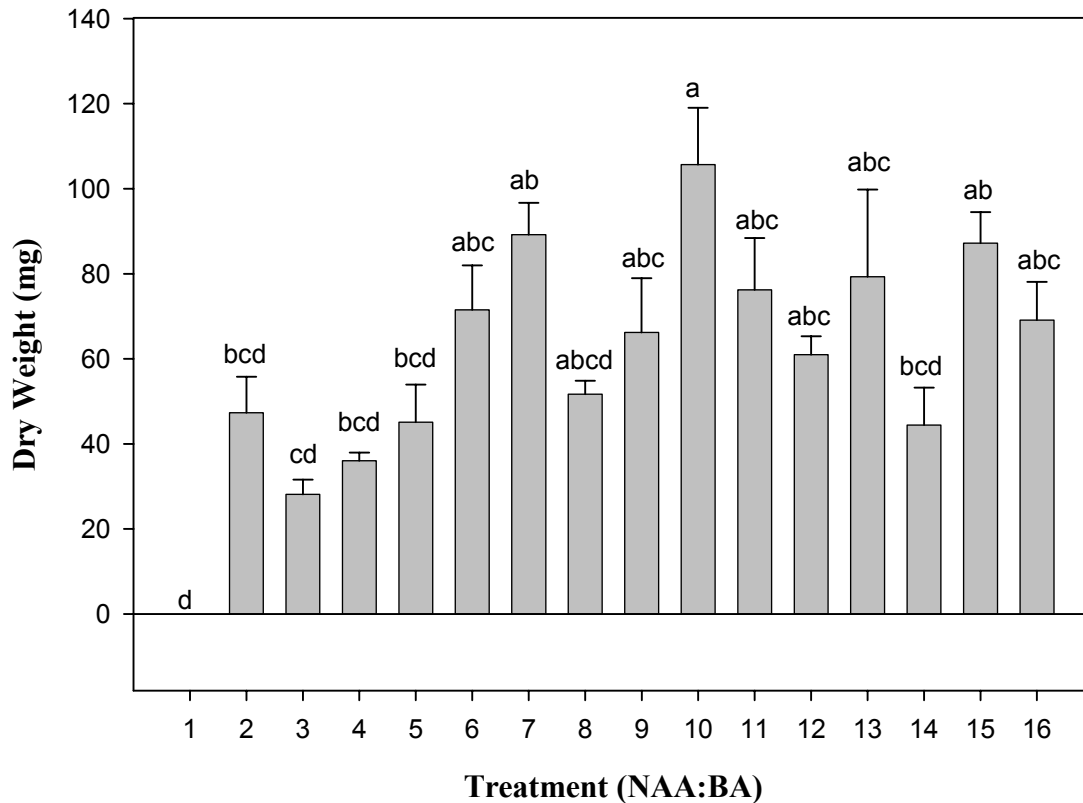


Figure 3.1 Mean dry weight (mg) of callus tissues. Treatments with the same letters are not significantly different at $p \leq 0.01$.

NAA and BA were both determined to have a significant effect on callus production (Table 3.1).

The interaction between the two effects also has a significant effect on callus production ($p=0.0008$).

Table 3.1 ANOVA for the effects of NAA and BA on callus production in *Eucommia ulmoides* explants.

Effect	Num DF	Den DF	Pr >F
NAA	3	48	<0.0001
BA	3	48	0.0049
NAA*BA	9	48	0.0008

HPLC analysis was used to detect the presence of PG and aucubin. It was found that there was no AU produced within the callus tissues, with only one sample of 120 containing AU. HPLC analysis of the samples for PG yielded more positive results. PG was detected in all treatments except for the control. Figure 3.2 depicts the results of how NAA and BA affect PG production. The treatments with 4 mg/l BA (BA 4) produced the highest amounts of PG at all NAA levels except for 9 mg/l NAA. At this level, 1 mg/l BA produced the second highest PG concentration. It was found that the ratio of 3 mg/l NAA to 4mg/l BA had the highest concentration of PG. Low BA levels alone did not produce PG, and in conjunction with NAA, produced PG concentrations that mostly were not significantly different from other treatments.

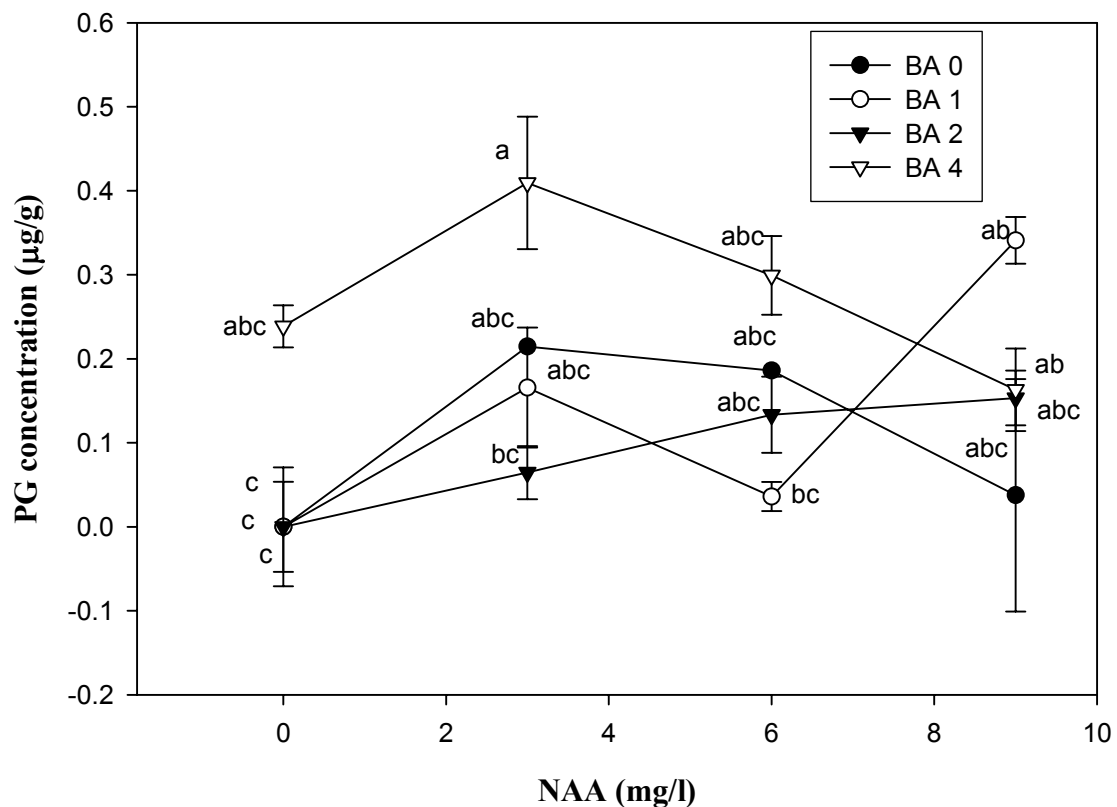


Figure 3.2 Graph showing the effects of NAA and BA on the production of pinoresinol diglucoside (PG) in *E. ulmoides* callus cultures. Means with the same letters are not significantly different at $p < 0.05$.

The main effects, NAA and BA, as well as the interaction between them, NAA*BA, were found to have a significant effect on PG production (Table 3.2).

Table 3.2 ANOVA for the effects of NAA and BA on the production of pinoresinol diglucoside (PG) in callus cultures produced from *Eucommia ulmoides* explants.

Effect	Num DF	Den DF	Pr > F
NAA	3	47	0.0021
BA	3	47	<0.0001
NAA*BA	9	47	0.0012

3.4 Discussion

The production of secondary metabolites in callus or suspension cultures can vary greatly. Usually, the accumulation of concentrations are lower or higher than concentrations found in vivo or fail to accumulate, both of which occurred in this experiment. Only PG was detected within the callus cultures of this experiment. Previous experiments have shown that PG, AU and other secondary metabolites can be produced in callus or suspension culture of *E. ulmoides* (Nakazawa and Toda, 1995; Tang et al., 1997; Tang et al., 2002; Wang et al., 2003; and Zhang et al., 1997). The absence of AU in the callus cells is not an unusual result, since the production of monoterpenes as well as other secondary metabolites in callus culture is often difficult to achieve. This is because in callus and suspension cultures cells are undifferentiated, which means the genes that are in control of secondary metabolite production may be turned off or not under specific control (Wink, 1986). The loss of AU production suggests that its biosynthesis pathway is organ specific and is linked to the level of differentiation of the callus culture (Becker, 1970; Brown and Charlwood, 1986; Wink, 1990). The biosynthesis of secondary metabolites is usually restricted to one tissue or a specific development stage, while accumulation can occur in all or most plant organs. Results from the previous chapter show that

while AU production is present in leaf and cortex samples, the cortex samples had higher AU concentrations (Table 2.3). Given this information, perhaps a callus culture derived from *E. ulmoides* cortex samples would result in aucubin production. Another explanation for the loss of AU production is that the nutritional environment can have a significant effect on secondary metabolite production (Becker and Sauerwein, 1986). In Nakazawa and Toda (1995), PG and AU were produced using MS medium, while this experiment used WPM medium. It is possible that the differences in composition of the two media types can explain the lack AU production, an idea worthy of further exploration.

Although the effects of PGRs on *Eucommia* plant tissues have been well documented, there is still much to be learned about the effects on callus cultures. In this experiment, NAA was added to the media in four levels: 0, 3, 6, and 9 mg/l. It was expected that as NAA concentrations increased, an inhibition of callus growth and therefore a reduction in PG concentration would occur. In fact, although there were significant differences in PG concentration and a reduction in PG production, none of the NAA only treatment levels inhibited callus growth. This is supported by Nakazawa et al. (1990), who found that using NAA alone could produce callus tissue. In their experiment, 100% of explants treated with 0.1 mg/l NAA initiated callus. However, this is in direct contrast to Tang et al. (1997), which reports that using NAA alone inhibits callus growth. This difference in results can be attributed to many factors. The concentration of NAA used by Tang et al. (1997), 0.8 mg/l, is a much lower concentration than the concentrations that were used in this experiment (0, 3, 6, and 9 mg/l). Also, Tang et al. (1997) used MS medium, which has a different composition than WPM, and thus different levels of minerals/nutrients. Different nutrient levels, along with PGR concentrations, temperature,

light, humidity, and plant genotype are factors that determine callus initiation and growth from plant tissues.

Using BA without an auxin supplement produced the same results as using NAA without a cytokinin supplement. Explants receiving 4 mg/l BA without NAA were the only treatment to produce PG (Figure 3.2), and the entire BA only treatments produced callus. BA did not seem to inhibit callus initiation, while PG concentration was inhibited. This is also supported by Nakazawa et al. (1990), in which, BA in levels of 0.1, 0.5, 1.0, 2.0, 2.5, 5.0, and 7.5 mg/l without NAA supplement produced callus in at least 73% of the explants, with 96% of the explants responding to 1.0 mg/l BA and 91.2% responding to 5 mg/l BA.

In this experiment, all of the treatment levels except for the control produced callus. Nakazawa and Toda (1995) used an even ratio of NAA to BA (0.5 mg/l) to induce callus and Tang et al. (1997, 2002) used a nearly even ratio (0.8 mg/l NAA to 0.6 mg/l BA) to induce callus. Zhu et al. (1997) used a low NAA concentration (0.5 mg/l) to a high BA concentration (2 mg/l) to induce callus. These findings indicate that nearly any concentration of PGRs will induce callus, but different concentrations will have an effect on secondary metabolite production. The results from this experiment show that 3 mg/l NAA to 4 mg/l BA gives the highest PG concentration (Figure 3.2). As concentrations increased, no difference in concentration was detected. More experimentation is needed to further optimize production of secondary metabolites in *E. ulmoides* callus culture.

CHAPTER 4. SUMMARY AND CONCLUSIONS

Eucommia ulmoides is a tree with many secondary compounds that have a variety of applications. Although secondary metabolites share similar pathways of biosynthesis with primary metabolites, their function is quite different. Unlike primary metabolites, secondary metabolites are not required for growth and development and are not equally distributed across taxonomic groups. They have various applications as natural products that include dyes, flavoring agents, waxes, oils, perfumes, and drugs. Secondary metabolites also have biological functions that aid in plant survival by protecting against herbivory and microbial infection, by acting as attractants for pollinating and seed-dispersing animals, and as alleopathic agents. This study focused on finding the seasonal changes in the concentrations of PG and AU of *E. ulmoides* trees cultivated in Baton Rouge, Louisiana as well as using tissue culture methods to observe how the above mentioned secondary metabolites (PG and AU) accumulate within callus culture.

The first experiment of this research observed the seasonal changes of PG and AU in the cortex and leaves of ten 3-year-old *E. ulmoides* trees during one growing season. TLC was used as a qualitative measure of four secondary metabolites: PG, AU, GE, and GA. PG was found in April leaf and cortex samples, but was not present in either sample type of the June, August, October, December, February, or April 2002 samples. GA was present in the June through April 2002 cortex samples and in the April 2001, June, and April 2002 leaf and cortex samples, while AU was shown to be present in all cortex samples and all leaf samples except for December and February.

HPLC was used to quantify and confirm the presence of PG and AU. PG was not detected in any of the monthly leaf and cortex samples. AU was detected in all cortex

samples and in the June, August, and April 2002 leaf samples. No significant difference was found in aucubin concentrations in *E. ulmoides* leaves, while the highest concentration of AU in cortex samples was found in the April 2001 and June samples. From this data, it appears that aucubin accumulates in the leaf and cortex during the spring and summer months. A study that focused on ecological factors such as rainfall, nutrient levels, stress, and temperature would be helpful in giving a more accurate description of how and when aucubin and PG accumulate in *Eucommia*.

The second experiment involved the induction of callus culture from *Eucommia* leaves. It has been shown that callus cells can be used to produce secondary compounds. The problem, however, is that secondary metabolite concentrations can vary greatly from the concentrations found naturally. The purpose of this experiment was to induce callus formation and analyze secondary metabolite production using HPLC. Four levels of the auxin NAA (0, 3, 6, and 9 mg/l) and four level of the cytokinin BA (0, 1, 2, and 4 mg/l) were used in this experiment. HPLC analyses of the resulting callus tissues showed that PG was produced and AU was not by the callus tissues. The concentrations of two plant regulators, NAA and BA, had significant effects on PG production. A ratio of 3 mg/l NAA to 4 mg/l BA produced the highest PG concentration.

The results from these experiments can be used to determine a harvesting scheme for *Eucommia*'s natural products. It may be best to collect leaves during the spring and summer, since aucubin was detected in leaf samples during this time period. Collecting leaves instead of cortex would provide a sustainable harvesting method that would not jeopardize the health of the trees over time. Although the cortex can be regenerated without harming the tree (Cui et al., 2000; Li et al., 1982; Li et al., 1983; and Zhang et

al., 1984), there is a lag time between harvesting and regeneration. Using the leaves as an alternate source for AU would present a faster turnaround time and could be more efficient.

The callus culture of *Eucommia* can be an alternative method for PG production. Although the literature shows that PG is found in *Eucommia* leaf and cortex (Deyama, 1983, Deyama et al., 1985, Deyama et al., 1986a, Duke, 1992; Hu, 1979; Sih et al., 1976), PG was not found in the *Eucommia* trees in cultivation in Baton Rouge, Louisiana. Callus cultures can be initiated and maintained using the information from this research and PG can then be extracted from the resulting tissue.

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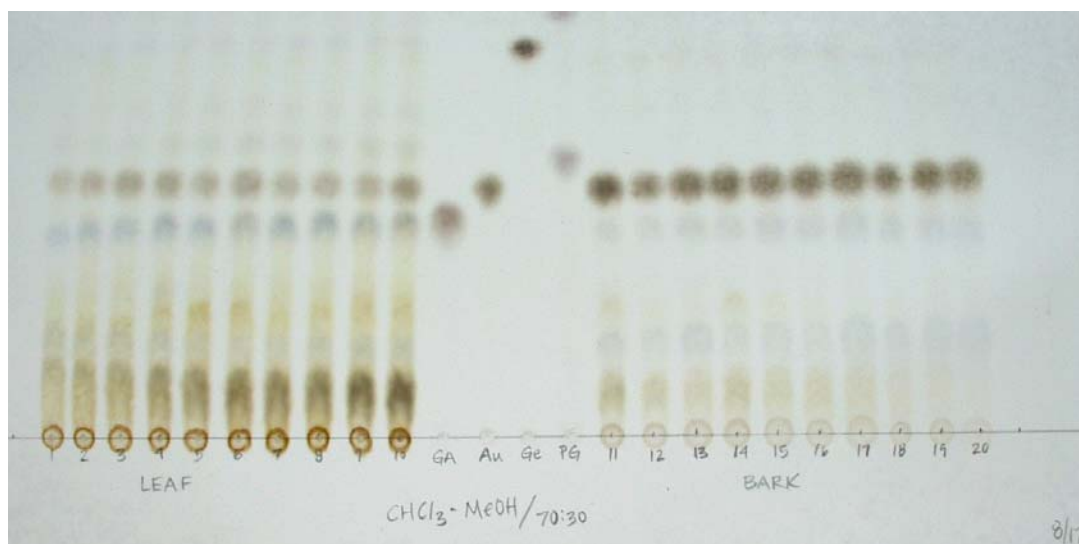
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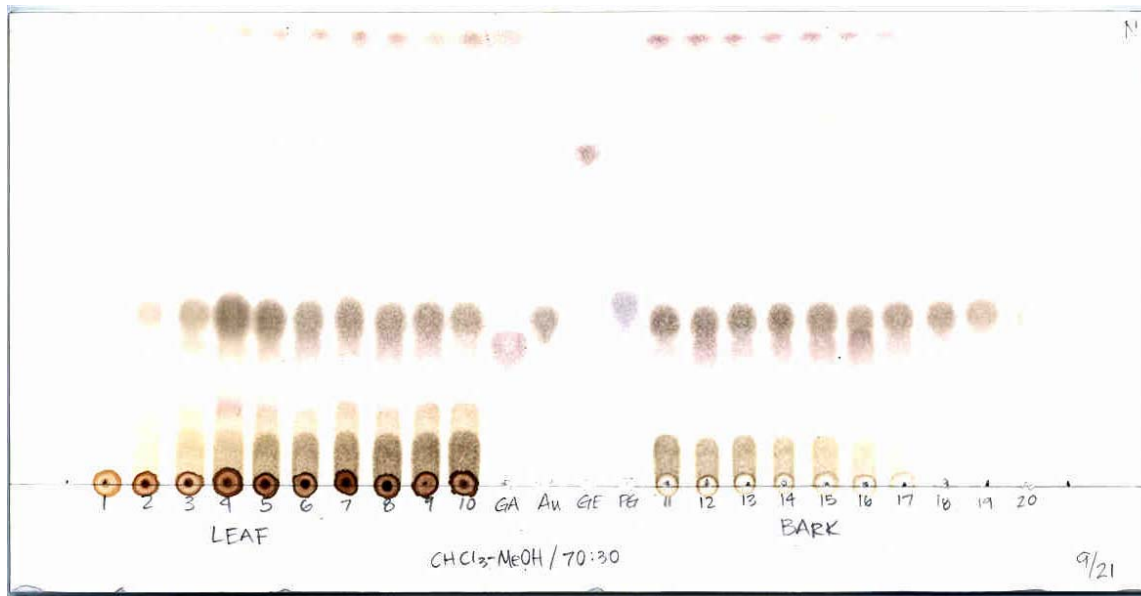
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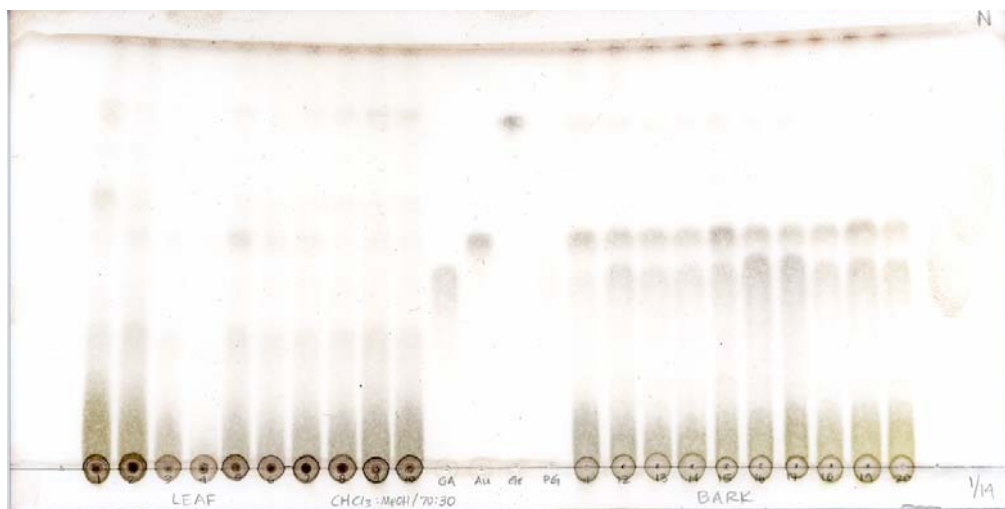
APPENDIX A: TLC PLATES



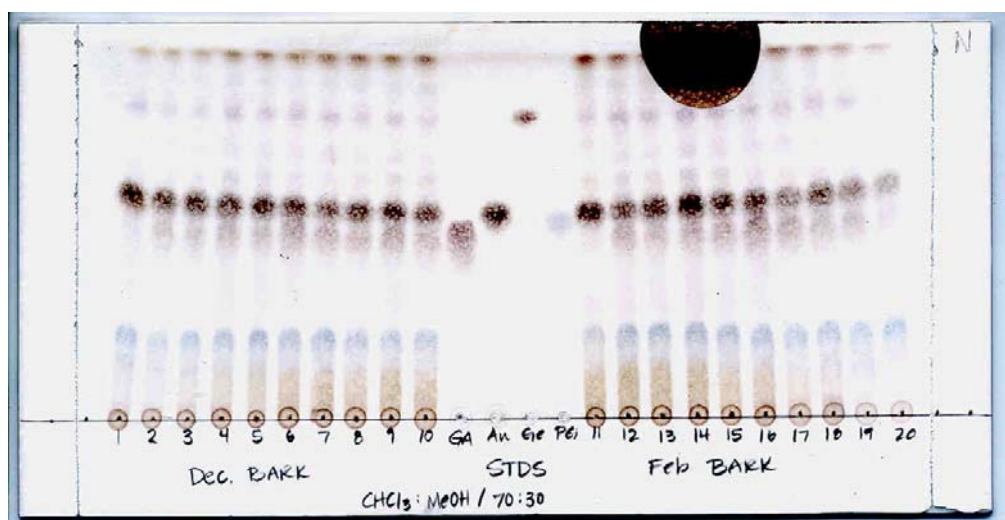
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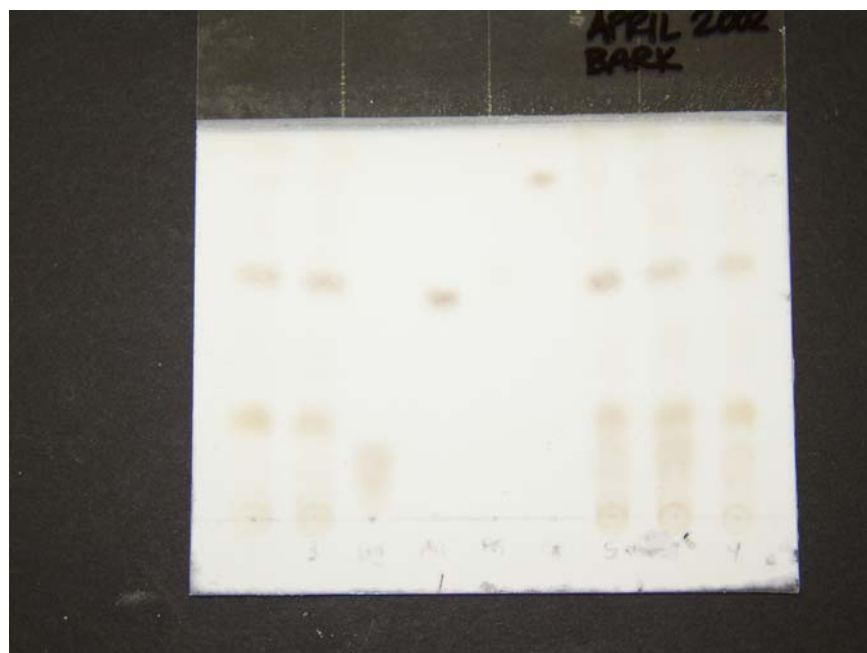
JUNE 2001



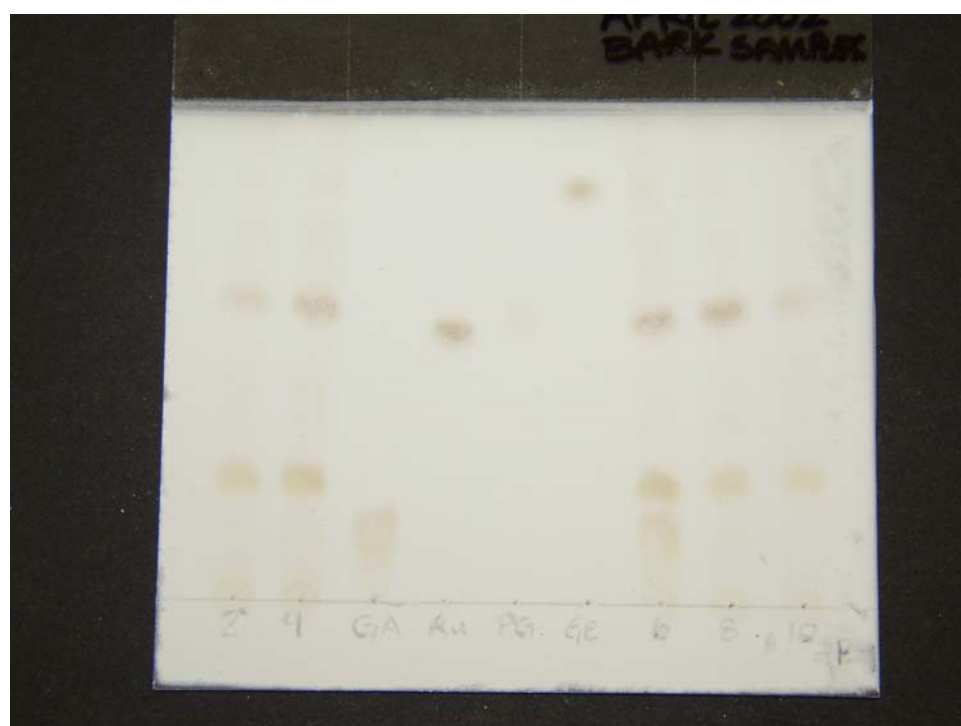
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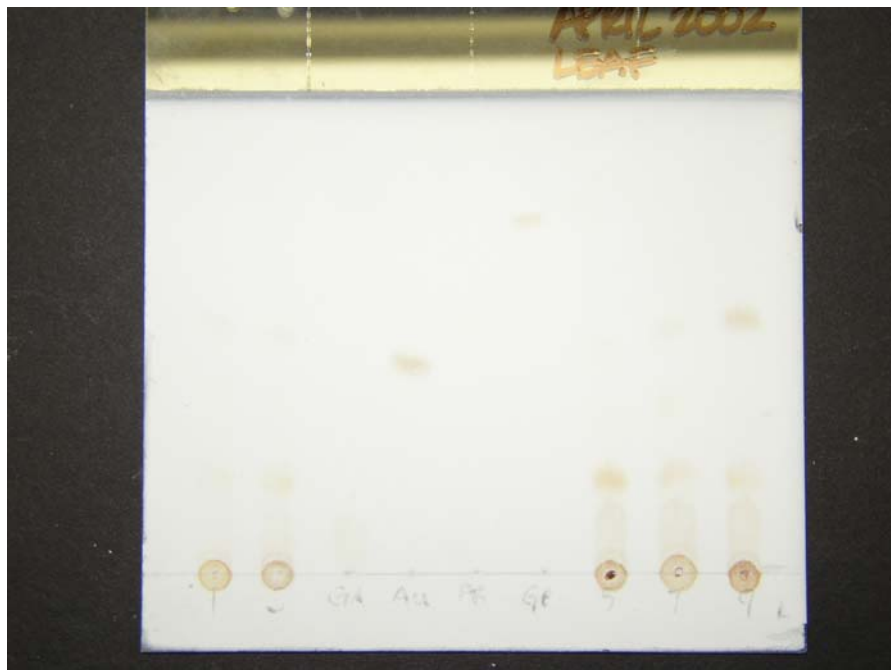
DECEMBER 2001/ FEBRUARY 2002



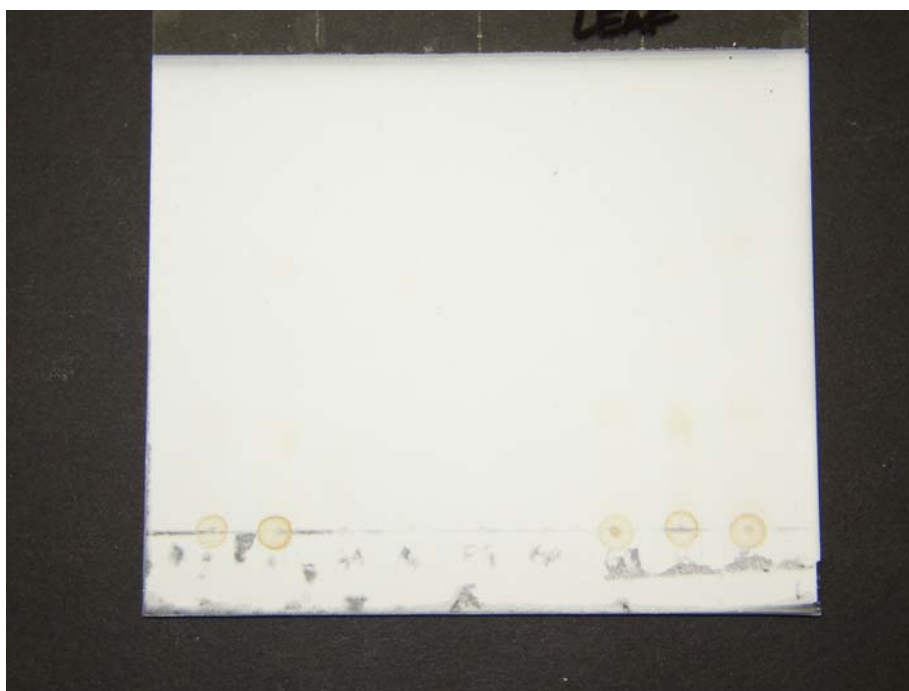
APRIL 2002 BARK (odd samples)



APRIL 2002 BARK (even samples)



APRIL 2002 LEAF (odd samples)



APRIL 2002 LEAF (even samples)

APPENDIX B: MONTHLY SAMPLE MEANS

Monthly aucubin concentrations (mean \pm Standard Error) in *Eucommia ulmoides* leaves and cortex. Means with different letters are significantly different at $p>0.05$ and a separated by type.

Type	Month	Aucubin ($\mu\text{g/g}$)
Leaf	April	$0.0 \pm 3.228\text{E-}6$ a
	June	$0.1086 \pm 3.857\text{E-}6$ a
	August	$0.0179 \pm 3.402\text{E-}6$ a
	October	$0.0 \pm 3.228\text{E-}6$ a
	April '02	$0.1260 \pm 3.228\text{E-}6$ a
Cortex	April	$1.416 \pm 9.578\text{E-}6$ a
	June	$1.061 \pm 9.578\text{E-}6$ a
	August	$0.6190 \pm 9.578\text{E-}6$ b
	October	$0.1530 \pm 9.578\text{E-}6$ c
	December	$0.3931 \pm 9.578\text{E-}6$ bc
	February	$0.4133 \pm 9.578\text{E-}6$ bc
	April '02	$0.1020 \pm 9.578\text{E-}6$ c

APPENDIX C: SAS PROGRAMS

SEASONAL CHANGES PROGRAM

```
dm 'log; clear; clear output;';
title1 'Aucubin Analysis of E.ulmoides Bark/Cortex';
options ls=78 ps=54;
data aucubin;
input treenum month$ type$ microg;
cards;
1    APRIL L    0
2    APRIL L    0
3    APRIL L    0
4    APRIL L    0
5    APRIL L    0
6    APRIL L    0
7    APRIL L    0
8    APRIL L    0
9    APRIL L    0
10   APRIL L    0
1    APRIL B    1.43188
2    APRIL B    0.841114
3    APRIL B    1.71802
4    APRIL B    1.13855
5    APRIL B    1.55295
6    APRIL B    1.58998
7    APRIL B    1.98748
8    APRIL B    1.44019
9    APRIL B    1.52542
10   APRIL B    0.938812
1    JUNE L    0
2    JUNE L    0
4    JUNE L    0.307554
5    JUNE L    0.136303
6    JUNE L    0.0959985
8    JUNE L    0.0716866
10   JUNE L    0.148957
1    JUNE B    1.35857
2    JUNE B    0.950015
3    JUNE B    1.59363
4    JUNE B    1.46325
5    JUNE B    0.81979
6    JUNE B    0.784929
7    JUNE B    1.17382
8    JUNE B    0.974342
9    JUNE B    0.889254
10   JUNE B    0.604641
1    AUG L    0
2    AUG L    0
3    AUG L    0
4    AUG L    0
5    AUG L    0.151352
6    AUG L    0.0101116
8    AUG L    0
9    AUG L    0
10   AUG L    0
```

1	AUG	B	0.469351
2	AUG	B	0
3	AUG	B	0.302989
4	AUG	B	0.511091
5	AUG	B	0.696163
6	AUG	B	0.673325
7	AUG	B	1.82292
8	AUG	B	0
9	AUG	B	0.927285
10	AUG	B	0.787092
1	OCT	L	0
2	OCT	L	0
3	OCT	L	0
4	OCT	L	0
5	OCT	L	0
6	OCT	L	0
7	OCT	L	0
8	OCT	L	0
9	OCT	L	0
10	OCT	L	0
1	OCT	B	0.184775
2	OCT	B	0.239923
3	OCT	B	0
4	OCT	B	0.393468
5	OCT	B	0.391727
6	OCT	B	0
7	OCT	B	0.319795
8	OCT	B	0
9	OCT	B	0
10	OCT	B	0
1	DEC	B	0.227454
2	DEC	B	0.155195
3	DEC	B	0.390778
4	DEC	B	0.37319
5	DEC	B	0.718649
6	DEC	B	0.659506
7	DEC	B	0.322217
8	DEC	B	0.299513
9	DEC	B	0.784544
10	DEC	B	0
1	FEB	B	0.906808
2	FEB	B	0.400343
3	FEB	B	0.544581
4	FEB	B	0.596748
5	FEB	B	0.284839
6	FEB	B	0.28171
7	FEB	B	0.122679
8	FEB	B	0.4895
9	FEB	B	0.295051
10	FEB	B	0.210837
1	APR2	L	0.0522766
2	APR2	L	0.082905
3	APR2	L	0
4	APR2	L	0.16682
5	APR2	L	0
6	APR2	L	0
7	APR2	L	0

8	APR2	L	0
9	APR2	L	0.513558
10	APR2	L	0.444444
1	APR2	B	0.140479
2	APR2	B	0.0702963
3	APR2	B	0.181653
4	APR2	B	0.13611
5	APR2	B	0.0991788
6	APR2	B	0.0550186
7	APR2	B	0.0664187
8	APR2	B	0.0989511
9	APR2	B	0.118563
10	APR2	B	0.0528478

```

;
proc sort data=aucubin; by type month; run;
proc means mean n stderr data=aucubin; by type month; var microg;
run;

proc mixed data=aucubin; by type;
  title2 'MIXED analysis without repeated and AR(1)';
  classes treenum month;
  model microg = month / HTYPE=3;
  random treenum;
  lsmeans month / cl adjust=tukey pdiff;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs ;* lsmeans;
;
proc mixed data=aucubin; by type;
  title2 'MIXED analysis with repeated and AR(1)';
  classes treenum month;
  model microg = month / HTYPE=3 outp=aucubinRES;
  random treenum;
  repeated month / subject=treenum type=AR(1);
  lsmeans month / cl adjust=tukey pdiff;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs ;* lsmeans;

%include 'a:pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05, sort=yes);

proc univariate data=aucubinRES normal plot;
  title2 'MIXED analysis residual analysis';
var resid;
run;
quit;

```

CALLUS CULTURE (PG) PROGRAM

```
dm'log; clear; clear output;';  
title Eucommia Callus Experiment;  
***missing data:5 2 3 0 .0328125***  
options ls=78 ps=54;
```

```
input trt rep NAA BA PG;  
cards;
```

1	1	0	0	0
1	2	0	0	0
1	3	0	0	0
1	4	0	0	0
2	1	0	1	0
2	2	0	1	0
2	3	0	1	0
2	4	0	1	0
3	1	0	2	0
3	2	0	2	0
3	3	0	2	0
3	4	0	2	0
4	1	0	4	.4444297082
4	2	0	4	.510380622
4	3	0	4	0
4	4	0	4	0
5	1	3	0	.099393019
5	3	3	0	.20125
5	4	3	0	.343253968
6	1	3	1	.231818181
6	2	3	1	.029655268
6	3	3	1	.335034013
6	4	3	1	.06638418
7	1	3	2	.082810539
7	2	3	2	.044392523
7	3	3	2	.10309973
7	4	3	2	.0276907
8	1	3	4	.370118845
8	2	3	4	.467105263
8	3	3	4	.35428051
8	4	3	4	.446315789
9	1	6	0	.255208333
9	2	6	0	.029411764
9	3	6	0	.255960729
9	4	6	0	.203316953
10	1	6	1	.130705394
10	2	6	1	.013368983
10	3	6	1	0
10	4	6	1	0
11	1	6	2	.015355086
11	2	6	2	.235346358
11	3	6	2	.147752809
11	4	6	2	.134963768
12	1	6	4	.391071428
12	2	6	4	.276487663
12	3	6	4	.23816568
12	4	6	4	.291262135

13	1	9	0	.110227272
13	2	9	0	.00389105
13	3	9	0	.005319148
13	4	9	0	.030737704
14	1	9	1	.252
14	2	9	1	.571174377
14	3	9	1	.226386806
14	4	9	1	.314417177
15	1	9	2	.067661691
15	2	9	2	.113038906
15	3	9	2	.284866468
15	4	9	2	.147780373
16	1	9	4	.081497797
16	2	9	4	.074666666
16	3	9	4	.257305194
16	4	9	4	.23877551

```

;
proc mixed data=callus CL ;
classes trt rep NAA BA;
model PG=NAA BA NAA*BA / outp=next1;
lsmeans NAA BA NAA*BA / adjust=tukey pdiff;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;

%include 'a:pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.01, sort=yes);

proc means mean n stderr data=callus;
by NAA BA;
var PG;
output out=callusmeans n=n mean=mean stderr=stderr;
run;
proc print data=callusmeans;
run;

proc univariate data=next1 normal plot;
var resid;
run;
quit;

```

CALLUS DRYWT (mg) PROGRAM

```
dm'log; clear; clear output;';  
title Eucommia Callus Experiment/drywt(mg);  
options ls=78 ps=54;
```

```
input trt rep NAA BA drywt;  
cards;
```

1	1	0	0	0
1	2	0	0	0
1	3	0	0	0
1	4	0	0	0
2	1	0	1	37.1
2	2	0	1	72.7
2	3	0	1	39.9
2	4	0	1	39.5
3	1	0	2	29.5
3	2	0	2	28.0
3	3	0	2	35.9
3	4	0	2	19.0
4	1	0	4	37.7
4	2	0	4	38.9
4	3	0	4	37.0
4	4	0	4	30.2
5	1	3	0	65.9
5	2	3	0	24.0
5	3	3	0	40.0
5	4	3	0	50.4
6	1	3	1	55.0
6	2	3	1	101.2
6	3	3	1	58.8
6	4	3	1	70.8
7	1	3	2	79.7
7	2	3	2	107.0
7	3	3	2	74.2
7	4	3	2	95.7
8	1	3	4	58.9
8	2	3	4	45.6
8	3	3	4	54.9
8	4	3	4	47.5
9	1	6	0	28.8
9	2	6	0	83.3
9	3	6	0	71.3
9	4	6	0	81.4
10	1	6	1	72.3
10	2	6	1	112.2
10	3	6	1	101.3
10	4	6	1	136.8
11	1	6	2	104.2
11	2	6	2	56.3
11	3	6	2	89.0
11	4	6	2	55.2
12	1	6	4	56.0
12	2	6	4	68.9
12	3	6	4	67.6
12	4	6	4	51.5

13	1	9	0	44.0
13	2	9	0	128.5
13	3	9	0	47.0
13	4	9	0	97.6
14	1	9	1	50.0
14	2	9	1	28.1
14	3	9	1	66.7
14	4	9	1	32.6
15	1	9	2	100.5
15	2	9	2	95.1
15	3	9	2	67.4
15	4	9	2	85.6
16	1	9	4	90.8
16	2	9	4	75.0
16	3	9	4	61.6
16	4	9	4	49.0

```

;
proc mixed data=callus CL ;
classes trt rep NAA BA;
model drywt=NAA BA NAA*BA / outp=next1;
lsmeans NAA BA NAA*BA / adjust=tukey pdiff;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;

%include 'a:pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.01, sort=yes);

proc means mean n stderr data=callus;
by NAA BA;
var drywt;
output out=callusmeans n=n mean=mean stderr=stderr;
run;
proc print data=callusmeans;
run;

proc univariate data=next1 normal plot;
var resid;
run;
quit;

```

APPENDIX D CALLUS TREATMENTS

<u>Treatment</u>	<u>NAA / BA (mg/l)</u>
1	0 / 0
2	0 / 1
3	0 / 2
4	0 / 4
5	3 / 0
6	3 / 1
7	3 / 2
8	3 / 4
9	6 / 0
10	6 / 1
11	6 / 2
12	6 / 4
13	9 / 0
14	9 / 1
15	9 / 2
16	9 / 4

VITA

Erica Deshay Gray was born on February 24, 1976 in Monroe, Louisiana to James and Edith Gray. She received a Bachelor of Science degree in Plant Biology from Louisiana State University in May 2000. Following an interest in tissue culture, she accepted a graduate assistantship in August 2000 in the School of Renewable Natural Resources at Louisiana State University. She is currently a candidate for the Master of Science degree in Forestry.