MICROPROPAGATION OF PECAN

By

BRENDA CAROL COTTEN

Bachelor of Science

in Agriculture

Oklahoma State University

Stillwater, Oklahoma

1981

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 1983

MICROPROPAGATION OF PECAN

Thesis Approved:

Thesis Adviser

Dean of the Graduate College

ACKNOWLEDGMENTS

The author wishes to express her gratitude to Dr. Johnny L. Johnson, her major adviser for his constant support, encouragement, and patience throughout the research and thesis completion. Appreciation is also extended to Dr. Michael Smith and Dr. Earl Mitchell for their assistance and cooperation.

Very special appreciation is expressed to my Mother and Father for all their assistance and support during my education.

Special thanks is extended to Mr. Scott Simmons for his patience throughout the writing of this thesis.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

The pecan, Carya illinoinensis (Wang), K. Koch, is propagated commercially by budding or grafting an adult scion onto seedling rootstocks. Pecan rootstocks are propagated by seed and are, therefore, heterozygous. Thus, due to genetic variability of the rootstock, pecan cultivars of proven and established quality may not perform consistently throughout an orchard. Clonal propagation of pecan would allow selection of rootstocks for specific genetic traits such as drought tolerance, production efficiency, salt tolerance, size control and mineral uptake. One way to proliferate the special genetic makeup of a superior rootstock would be through the mass clonal propagation of that particular rootstock. However, pecan resists traditional vegetative propagation methods once the adult phase of growth is well established. As a tree approaches maturity and its quality and performance have been determined, it becomes very difficult or impossible to root the cuttings on a commercial scale. However, cuttings taken from a tree still in its juvenile stage of growth will root with

somewhat more success although still not commercially significant. Tissue culture methods of propagation offer a way to increase commercial production by generating a larger number of plants produced in a shorter amount of time than could be obtained with rooted cuttings. A method of micropropagating clonal pecans could also produce improved cultivars established on their own root systems, which would be important from a commercial production standpoint. A clonal scion cultivar established on its own root system would eliminate the time and cost spent budding and grafting onto seedling rootstocks.

Experimental methods have been established for the micropropagation of pecans using juvenile tissue (15). However, a problem still exists. That is, by using juvenile tissue there is the uncertainty of how this plant will eventually perform under field conditions. There are no means to determine whether or not the seedling will be of superior quality until it has been placed in an orchard and brought into production. Therefore, it would be advantageous to use adult pecan tissue of known performance for micropropagation. By using adult tissue, it would be possible to selectively choose a tree, whose performance is superior under certain environmental conditions, and utilize tissue from this tree for mass

clonal propagation. However, using adult tissue for micropropagation is not without its disadvantages.

Previous studies have attempted micropropagation techniques utilizing adult tissue (21). These studies have shown that not only is there contamination on the external surface of the tissue, but contaminants are inherent to the internal tissues as well (20). Previous attempts at sterilization of these internal contaminants have not been successful (62). One problem associated with establishing a sterilization technique is there seems to exist a very fine line between being able to control the internal contamination and killing the host tissue.

The objectives of this study were to: 1) determine sterilization techniques for controlling internal as well as external contamination for the micropropagation of adult pecan tissue, and 2) to establish correct media requirements (e.g. hormones) necessary for the eventual cloning of adult pecan tissue.

CHAPTER II

LITERATURE REVIEW

Tissue Culture

'Plant tissue culture' is a general term used to describe the aseptic cultivation, in vitro, of all plant tissues, whether from a single cell, tissue, or organ. A more restricted definition of the term has been presented by Street (54). Plant cell, tissue and organ culture consist of isolating a cell, tissue or organ and aseptically placing it into a container having a specific nutrient medium, under controlled environmental conditions (light, temperature, humidity, etc.), with the objective of obtaining rapid asexual multiplication of plant cells or plants. Theoretically, any given tissue contains cells that have all the genetic information required to produce the many and various types of cells which make up an intact plant. Some of the plant parts used include shoot tips, axillary buds, stem sections, leaf sections, microspores, megaspores, ovules, embryos, seeds, as well as isolated cells and protoplasts. Plant tissue culture

techniques using the above mentioned tissues can be used in studying organ formation (43), cell and tissue differentiation (49, 50, 51, 52), and organ growth and differentiation (59). Not only has plant tissue culture been important in experimental studies dealing with plant development, it also is used for the production of pharmaceuticals and other natural products (63, 64), hybridization and development of new cultivars (44), establishment of disease-free stock (34), preservation of germplasm (19), and rapid clonal multiplication of desirable plants (9). These developments in tissue culture have evolved through many years of experimental successes and failures.

Plant propagation through tissue culture dates back as early as 1902 when Haberlandt first attempted to regenerate plants from single cells (14, 22). Although this early work was unsuccessful, it established a conceptual framework upon which all current tissue culture research is based. Research in tissue culture was slow until 1934 when White (59) successfully established an actively growing clone of tomato roots. In 1939, Nobecourt (32) and Gautheret (11) independently established the first continuous culture of callus using carrot. About the same time Nobecourt and Gautheret reported their findings, White (60) reported identical results using tobacco plants. These experiments represent

the first true plant tissue cultures in the strict sense of maintaining prolonged cultures of unorganized cells. By 1957, Skoog and Miller (43) demonstrated that the ratio of auxin to cytokinin would induce callus tissue cultures to differentiate into intact plants with leaves and roots. Using a defined medium, Vasil and Hildebrandt (57) differentiated roots and shoots with leaves from tobacco callus. The rooted shoots were placed into pots containing soil and transferred to a greenhouse where they grew into normal, healthy plants which eventually flowered.

During 1963, Wimber (61) reported a method for reproducing orchids using shoot meristems. Meristems from hybrid orchids were placed into agitated flasks of liquid media which allowed for the rapid growth of tissue from the shoot meristems. After a period of a few months, the tissue mass was divided into segments and placed onto a solid nutrient media for the formation of shoots. Using this method, Wimber was able to increase the number of plants obtained while controlling virus contamination. The success of Wimber (61) and others (27, 28, 38) led to the first commercial application of tissue culture using orchid meristems (35). Orchid plants that were at one time only increased 10 to 15 fold from one plant source with conventional propagation methods, were now being successfully increased 3,000 fold using plant tissue

culture techniques. These techniques used for rapid production of orchids are the first examples of tissue culture being used commercially on a routine basis.

In the early 1970's, Takebe et al (56) developed, for the first time, intact plants from isolated mesophyll protoplasts of of tobacco. The protoplasts were then stimulated to fuse under defined experimental conditions (33). Using the results of Takebe et al(56), Carlson et al(5) succeeded in fusing protoplasts isolated from two different species of tobacco. This experiment was significant in that it showed that it was possible to fuse protoplasts from two different plant species resulting in hybrid cells combining plant genotypes that cannot normally be crossed sexually.

These early experiments exemplify only a few of the more important advances that have contributed to the expansion of tissue culture. Tissue culture has evolved into a dynamic new field since the first attempts made by Haberlandt. However, the application of previous techniques, as well as new techniques, and their application to a variety of plant species is sure to result in further expansion of the field.

Woody Plants

The majority of the successes in propagation, using tissue culture methods, have been with the herbaceous

species. Tissue culture of woody plants is still in its beginning stages compared to the advances made using other species. Tissue culture of trees is often set apart from the culture of other species. Problems often associated with propagation of woody plants through tissue culture are not always encountered with herbaceous plants. Some of the major difficulties in the culture of woody plants include: 1) many trees are highly heterogeneous which can cause culture responses to be variable; 2) juvenile tissue of woody plants is generally more responsive than adult tissue of woody plants; 3) it is often difficult to obtain uncontaminated cultures from mature tissue, even after using sterilization procedures; and 4) it is often difficult to produce roots and shoots from callus of woody plants (40). Although woody plants, in general, are not as responsive to tissue culture techniques, the possibility does exist that tissue culture will develop into a practical means for the mass propagation of trees. Some of the objectives for developing methods for micropropagation of woody species include conservation of rare, endangered or slowly reproducing species, progeny testing and multiplication of desirable genotypes. Also, propagation of fruit and forest trees and ornamental woody species could have commercial applications to the nursery industry.

A faster method for propagating fruit trees would be desirable because of increased demand for fruit trees created by favorable prices, high density plantings, and changing cultivar preferences. For example, peach trees are normally propagated by budding, but this is costly and very time consuming. Recently, many growers have started using high density plantings of small peach trees to reduce labor costs while increasing yield potential (8). To reduce the costs of budding trees for high density plantings, trees could be produced on their own roots by cuttings or micropropagation. Micropropagation has the advantage over rooting cuttings in that the multiplication rate can be increased substantially (29). Recent reports have shown that tissue culture techniques can be used to propagate some peach cultivars (40, 41, 42) and other Prunus species (17, 31, 39, 42, 55).

Apple is another fruit tree which can benefit from the commercial application of tissue culture techniques. Micropropagation of apple has been most successful with juvenile seedlings (24), and apple rootstocks (16, 47, 58). Apple seedlings and rootstocks are easier to root by conventional methods than most scion cultivars. Although propagation by cuttings for most scion cultivars is difficult, there have been reports on the successful regeneration of plants using tissue culture techniques(1, 18, 48, 65).

A few of the other woody plants that have been successfully propagated by tissue culture methods include Pear (25), Western Hemlock (7), Sycamore (23), Olive (37), Elm (10), Chestnut (36), Boganvillea (6), Rhododendron (2), Citrus (4), and some forest tree species (12, 13).

The woody plants comprise a major group of commercially valuable plant species, and many cannot be propagated rapidly enough using conventional propagation techniques. Thus, there is a great need for expanding applications of tissue culture to more woody species to allow for the rapid production of true-to-type cloning of commercially valuable woody plants.

Pecan Micropropagation

Of all the attempts made at propagating woody plants through tissue culture techniques, pecan seems to be one of the most difficult. One method of obtaining clonal tissue of pecans, other than using micropropagation techniques, is to root stem cuttings. This method has yielded limited success (3, 26, 46, 53). It was determined that cuttings taken during different months of the year would give variable responses, and that juvenile cuttings root more readily than adult cuttings. Smith and Chiu (46) obtained optimum rooting percentages of up to 71% rooting of juvenile cuttings during February compared to 33% rooting with adult cuttings taken in August. They

interpreted these results as indicating that foliage must be present for rooting to occur. They further suggested the presence of a root-promoting substance synthesized in the leaves.

Since propagation of clonal pecans by rooted cuttings does not lend itself to commercial applications, attempts have been made to micropropagate them using tissue culture techniques. In an early experiment, Smith (45) attempted to micropropagate seedling pecans but was successful in developing only a few single roots from callus cultures. Using juvenile tissue, Knox and Smith (21) reported limited success, producing plantlets in 7 to 14% of the cultures. Although some plants were developed, the media components were not sufficiently defined to allow for the establishment of a substantial root system. Consequently, no plantlets became established when transferred to soil. Wood (62) has produced shoots from axillary buds of nodal explants from 4 to 12 week old 'Stuart' seedlings using a combination of 6-benzylamino purine (BA) and indolebutryic acid (IBA). Although proliferation of shoots was obtained, there was no report of root initiation. Later, Hansen and Lazarte (15) developed a successful method for <u>in vitro</u> propagation of juvenile seedling pecans using stem segments with lateral buds. Normal shoot development and 75% rooting were obtained. Those plantlets which

rooted were then successfully transferred to soil in the greenhouse.

Although root and shoot proliferation have been obtained using juvenile tissue, there have been no reports of successful *in vitro* propagation of adult pecan tissue. One major obstacle in obtaining cultures of adult tissue is the inherent contamination of the internal surfaces of tissue from field-grown trees (20). Previous attempts to eradicate these internal contaminants have as yet, proven unsuccessful. Thus, the focus of the present research is to define the techniques necessary for the sterilization of internal surface contaminants associated with adult tissue of pecan.

CHAPTER III

MATERIALS AND METHODS

Plant material for this research was collected in 1982-83 from 14-year old 'Mohawk' and 26-year old 'Stuart' trees growing at the Oklahoma Pecan Research Station near Sparks, Oklahoma. Explant material was selected from dormant terminal buds or terminal shoots depending on the time of year. Stem cuttings 3 to 9 cm long, were collected, placed in plastic bags containing a moist cloth and transported in a portable cooler to laboratory facilities at Stillwater, Oklahoma.

Stem segments were then prepared for sterilization by trimming the stems to an approximate length of 2 cm. When dormant buds were used, the shortened stem segment was stripped of all lateral buds. The bud scales surrounding the terminal bud were removed leaving the innermost 4 to 6 leaf primordia protecting the terminal apical meristem. If the time of year permitted using expanding shoots, then the shortened stem segment was stripped of all excess leaves, and the shoot was dissected leaving 4 to 6 leaf primordia protecting the terminal apical meristem. Stem

sections were then placed into beakers of distilled, deionized water with Tween 20 and agitated for 5 to 10 minutes to remove external dirt and residue. If the experiment was to use a benomyl soak, stem segments were transferred from the distilled-deionized water into a saturated benomyl solution with Tween 20 and agitated for various periods of time. The benomyl was rinsed from the stem segments with distilled, deionized water. Whether or not a benomyl soak was used, stem sections were then placed in sterile petri dishes and transferred to a laminar flow hood for further sterilization. All sterilization treatments included Tween 20 emulsifier to reduce surface tension and aid in penetration of the sterilizing solution into the tissues.

Following surface sterilization, the tissues were rinsed once in sterile, distilled, dionized water with Tween 20, then 3 to 4 rinses with sterile, distilled, deionized water excluding Tween 20. The explant was dissected to the apical meristem with 2 to 3 leaf primordia. The dissected apical meristem was excised from the stem segment and placed in 20 mm x 150 mm test tubes containing 25 ml slanted agar media.

Culture tubes containing explants were kept in a controlled environment growth chamber. Cultures were maintained at a temperature between 25°C and 28°C under 16-hour illumination.

At the termination of each experiment, data was collected from each explant. For the majority of the experiments, data was collected two weeks from the day of innoculation. Subsequent collection dates were determined by the nature of the experiment. Data collected generally consisted of % contamination, necrosis indexes, and growth indexes. The necrosis index was a visual rating from 0 to 5.

0=No necrosis

1=Tip of meristem necrotic

2=1/2 of meristem necrotic

3=All of meristem necrotic

4=Most of explant necrotic

5=Total necrosis, death of explant

The growth index was also a visual rating from 0 to 5.

0=Death of explant

1=Browning of explant

2=Yellowing of explant

3=Green apex

4=Swollen apex tissue

5=Shoot apex expansion

Media Composition

The media used in the majority of the experiments consisted of a variation of Murashige and Skoog's (30) high salts media.

Macronutrients	mg/liter
NH_4NO_3	1650
KNO_5	1900
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
Na-EDTA	37
$FeSO_4 \cdot 7H_2O$	27

Micronutrients	mg/liter
H_3BO_3	6.2
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 4H_2O$	8.6
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025

Vitamins	mg/liter
Nicotinic acid	1
Thiamine HCl	10
Pyridoxine HCl	1
Myo-inositol	100

Calcium Chloride	mg/liter
$CaCl_2 \cdot 2H_2O$	150

Potassium iodine	mg/liter
KI	0.75

Other media components	g/liter
Sucrose	30
Agar (Sigma)	6

Distilled-deionized water was used to bring the media to volume.

Growth regulator stock solutions were either dispensed with ul pipettes into individual test tubes or directly into one liter of media using ml pipettes at a rate dependant upon the experiment. To make growth regulator stock solutions, all growth regulators used were dissolved with 5.0 N KOH, and brought to volume with distilled, deionized water.

The media was adjusted to a pH of 5.8 with either 5.0 N KOH or 0.10 N HCl after all components had been added, except agar and charcoal (if used). The media was heated to a quick boil to dissolve the agar. Media was distributed into 25 mm x 150 mm Pyrex culture tubes at a rate of 25 ml/tube. Bellco Kap-uts were used as tube closures. Autoclaving was at 121^{o}C and 15 psi for 15 minutes. The culture tubes containing media were cooled at a 45^{o} angle.

Unless otherwise indicated, all experimental procedures were conducted as indicated above.

Experiments

Phenolics

A preliminary experiment utilizing apical meristems from mature pecan trees indicated that explant tissues exuded a high amount of apparent phenolic compounds which were toxic to the explant, causing death of the tissue

within a matter of days. Thus, an experiment was conducted attempting to control the amount of phenolic-like compounds released into the media surrounding the explant.

Experiment I - Polyvinylpolypyrrolidone, Activated Charcoal, and Darkness. This experiment was conducted to test the effects of polyvinylpolypyrrolidone (PVP), activated charcoal, and darkness on controlling apparent phenolic compounds. Activated charcoal and PVP were tested for their ability to absorb apparent phenolics released from the explant tissue. The dark treatment consisted of placing the explants in darkness for one week followed by exposure to light for the duration of the experiment, 4 to 5 weeks. The dark treatment was to reduce the amount of phenolic-like compounds released from the explant tissue. Apical meristems dissected from terminal shoots of 'Mohawk' pecans were used as explants. Explants were given a 24-hour soak in a saturated benomyl solution then surface sterilized with a 0.525% sodium hypochlorite solution for 15 minutes. The media used in all treatments contained 1.0 mg/liter of 6-benzylamino purine (BA). Each treatment consisted of 10 replications. The additions to the culture media and additional treatments consisted of:

1. PVP (3 g/liter)
2. PVP (3 g/liter), darkness

3. Activated charcoal (3 g/liter)
4. Activated charcoal (3 g/liter), darkness
5. PVP (3 g/liter), activated charcoal (3 g/liter)
6. PVP (3 g/liter), activated charcoal (3 g/liter), darkness
7. Darkness
8. No treatments

Experiment II - Liquid Media Soak, and Sterile, Distilled, Deionized Water Soak. Apical meristems dissected from terminal shoots of 'Mohawk' pecans were used to test the effectiveness of either liquid culture media or sterile, distilled, deionized water as soak treatments to absorb apparent phenolics released from the explant tissue before the explant was placed into culture tubes. Media used in all treatments contained 1.0 mg/litre of BA. Explants were subjected to a 24-hour soak in a saturated benomyl solution then surface sterilized for 15 minutes with 0.525% sodium hypochlorite. Each treatment consisted of five replicates. There were eight treatments consisting of:

1. Sterile, distilled, deionized water soak
2. Liquid media soak
3. Sterile, distilled, deionized water soak, darkness
4. Liquid media soak, darkness
5. Sterile, distilled, deionized water soak, activated charcoal (3 g/liter)

6. Liquid media soak, activated charcoal (3 g/liter)
7. Sterile, distilled, deionized water soak, PVP (3 g/liter)
8. Liquid media soak, PVP (3 g/liter)

Experiment III - Charcoal and Darkness. Apical meristems, dissected from terminal shoots of 'Stuart' pecans were used to test combinations of activated charcoal and one week dark treatments (as described in Experiment I) for reducing apparent phenolics. The media used in all treatments contained 1.0 mg/liter of indolebutryric acid (IBA) and 1.0 mg/liter of 6-benzylamino purine (BA). Explants were surface sterilized for 15 minutes in 0.525% sodium hypochlorite. Each treatment was replicated 20 times. Four combinations of treatments were used including:

1. Activated charcoal incorporated into the media (3 g/liter), no dark treatment,
2. Activated charcoal incorporated into the media (3 g/liter), darkness,
3. No activated charcoal, darkness,
4. No activated charcoal, no dark treatment.

Sterilization

One of the difficulties in obtaining cultures of adult pecan tissue is explants collected from field-grown trees are exposed to numerous fungal and bacterial contaminants. Not only is there contamination of the

external surface, but there are also contaminants inherent to the internal tissues of adult pecan. The following experiments were used to reduce external as well as internal contamination using various sterilization treatments.

Experiment IV - Antibiotics. To control bacterial and fungal contamination, explants were subjected to various combinations of antibiotic soaks and antibiotics incorporated into the culture media. Explants used were meristems dissected from dormant terminal buds of 'Mohawk' pecans. The media was supplimented with 1.0 mg/liter of BA and 3 g/liter activated charcoal. All explants were given a dark treatment and a "standard" surface sterilization of 0.525% sodium hypochlorite for 15 minutes. The duration for all soak treatments was 30 minutes. Explant material was collected on September 12, 1982. Treatments were replicated 5 times each. Treatments were as follows:

Incorporated into the Media	Soak Treatments
1. No bactericide or fungicide incorporated into the media	Standard surface sterilization
2. Gentamicin sulfate (bacteria) 50 mg/l	Standard Surface sterilization
3. Gentamicin sulfate (bacteria) 50 mg/l	Standard surface sterilization & Gentamicin sulfate 100 mg/l
4. Gentamicin sulfate (bacteria) 50 mg/l	Standard surface sterilization & Nystatin 100 mg/l

5. Nystatin (fungal & yeasts) 50 mg/l	Standard surface sterilization
6. Nystatin (fungal & yeasts) 50 mg/l	Standard surface sterilization & Gentamicin sulfate 100 mg/l.
7. Nystatin (fungal & yeasts) 50 mg/l	Standard surface sterilization and Nystatin 100 mg/l.
8. Penicillin - G (bacteria) 62.5 mg/l	Standard surface sterilization
9. Penicillin - G (bacteria) 62.5 mg/l	Standard surface sterilization & Gentimicin sulfate 100 mg/l.
10. Penicillin - G (bacteria) 62.5 mg/l	Standard surface sterilization & Nystatin 100 mg/l.
11. Streptomycin sulfate (bacteria) 100 mg/l	Standard surface sterilization
12. Streptomycin sulfate (bacteria) 100 mg/l	Standard surface sterilization & Gentamicin sulfate 100 mg/l.
13. Streptomycin sulfate (bacteria) 100 mg/l	Standard surface sterilization & Nystatin 100 mg/l.
14. Penicillin - G & Streptomycin sulfate	Standard surface sterilization.
15. Penicillin - G & Streptomycin sulfate	Standard surface sterilization & Gentamicin sulfate 100 mg/l.
16. Penicillin - G & Streptomycin sulfate	Standard surface sterilization & Nystatin 100 mg/l.

Experiment V - Benomyl Soaks. Apical meristems dissected from terminal shoots of 'Mohawk' pecans were used to test the effects of varying durations of benomyl soaks on the reduction of fungal contamination. The media was supplimented with 1.0 mg/liter BA and 1.0 mg/liter IBA. Explants were given a "standard" surface sterilization treatment of 0.525% sodium hypochlorite for 15 minutes. All soaks consisted of a saturated benomyl solution. Explant material was collected on July 9, 1982. Treatments were replicated 7 times. The treatments were:

1. 3-hour benomyl soak
2. 6-hour benomyl soak
3. 12-hour benomyl soak
4. 24-hour benomyl soak
5. No benomyl soak.

Experiment VI - Ultra Violet Light, Benzalkonium Chloride, Ethyl Alcohol, Sodium Hypochlorite. In order to reduce contamination of explant cultures, 10 combinations of 70% ethyl alcohol (ETOH), 0.525% sodium hypochlorite (NaOCl), ultra violet light (UV), and benzalkonium chloride (BKC) were utilized. Ultra violet light was supplied by a UV lamp in a laminar flow hood. The media was supplimented with activated charcoal (3 g/liter) and 1.0 mg/liter BA. All cultures were subjected

to a dark treatment. Treatments were replicated 8 times. Sterilization treatments consisted of:

1. 70% ETOH (3 min.)
2. 0.525% NaOCl (10 min.)
3. 0.525% NaOCl (15 min.)
4. 0.525% NaOCl (20 min.)
5. 70% ETOH (3 min.), 0.525% NaOCl (10 min.)
6. UV light (2 hrs.) 70% ETOC (3 min.)
7. UV light (2 hrs.), 0.525% NaOCl (10 min.)
8. UV light (2 hrs.), 0.525% NaOCl (10 min.), 70% ETOH (3 min.)
9. BKC (1 g BKC/liter of 0.525% NaOCl) for 5 min.
10. BKC (1 g BKC/liter of 0.525% NaOCl) for 5 min., 0.525% NaOCl (10 min.), 70% ETOH (3 min.).

Experiment VII - Sodium Hypochlorite and Ethyl Alcohol. Apical meristems, dissected from terminal shoots of 'Mowawk' pecans, were used to test combinations of 0.525% sodium hypochlorite (NaOCl) and 70% ethyl alcohol (ETOH) to reduce contamination. All treatments were soaked for 5 1/2-hours in a saturated benomyl solution and given darkness for one week. Media was supplimented with 1.0 mg/liter BA, and 3 g/liter activated charcoal. Explant material was collected on November 5, 1982. Treatments were replicated 5 times. There were eight different combinations of treatments consisting of:

1. 0.525% NaOCl (10 min.), 70% ETOH (5 min.)

2. 0.525% NaOCl (10 min.), 70% ETOH (10 min.)
3. 0.525% NaOCl (10 min.), 70% ETOH (20 min.)
4. 0.525% NaOCl (20 min.), 70% ETOH (5 min.)
5. 0.525% NaOCl (20 min.), 70% ETOH (10 min.)
6. 0.525% NaOCl (20 min.), 70% ETOH (20 min.)
7. 0.525% NaOCl (30 min.), 70% ETOH (5 min.)
8. 0.525% NaOCl (30 min.), 70% ETOH (10 min.)

Experiment VII - Sodium Hypochlorite and Ethyl Alcohol. This experiment was conducted to reduce contamination and determine the point of necrosis for eight separate treatments of 70% ethyl alcohol (ETOH) or 0.525% sodium hypochlorite (NaOCl) under different sterilization time periods. Explants were taken from meristems of terminal shoots of 'Mohawk' pecans. The media was supplimented with 1.0 mg/liter BA and activated charcoal (3 g/liter). Explants were subjected to a benomyl soak for 4 1/2 hours and a dark treatment. Explant material was collected on November 9, 1982. Treatments were replicated 5 times. Treatments were:

1. 70% ETOH (10 min.)
2. 70% ETOH (15 min.)
3. 70% ETOH (20 min.)
4. 70% ETOH (25 min.)
5. 0.525% NaOCl (20 min.)
6. 0.525% NaOCl (25 min.)

7. 0.525% NaOCl (30 min.)
8. 0.525% NaOCl (40 min.)

Experiment IX - Sodium Hypochlorite, Ethyl Alcohol. This experiment tested various sterilization time periods for combinations of 70% ethyl alcohol (ETOH) and 0.525% sodium hypochlorite (NaOCl). Explants used were apical meristems dissected from 'Mohawk' pecans. Ammendments to the media were 3 g/liter of activated charcoal and 1.0 mg/liter of BA. Pretreatments for all explants consisted of a dark treatment and a 4-hour benomyl soak. Explant material was collected on November 17, 1982. Treatments were replicated 5 times. The treatment consisted of:

1. 70% ETOH (4 min.), 0.525% NaOCl (10 min.)
2. 70% ETOH (4 min.), 0.525% NaOCl (15 min.)
3. 70% ETOH (4 min.), 0.525% NaOCl (20 min.)
4. 70% ETOH (4 min.), 0.525% NaOCl (25 min.)
5. 70% ETOH (6 min.), 0.525% NaOCl (10 min.)
6. 70% ETOH (6 min.), 0.525% NaOCl (15 min.)
7. 70% ETOH (6 min.), 0.525% NaOCl (20 min.)
8. 70% ETOH (6 min.), 0.525% NaOCl (25 min.)
9. 70% ETOH (8 min.), 0.525% NaOCl (10 min.)
10. 70% ETOH (8 min.), 0.525% NaOCl (15 min.)
11. 70% ETOH (8 min.), 0.525% NaOCl (20 min.)
12. 70% ETOH (8 min.), 0.525% NaOCl (25 min.)
13. 70% ETOH (10 min.), 0.525% NaOCl (10 min.)

14. 70% ETOH (10 min.), 0.525% NaOCl (15 min.)
15. 70% ETOH (10 min.), 0.525% NaOCl (20 min.)
16. 70% ETOH (10 min.), 0.525% NaOCl (25 min.)

Experiment X - Sodium Hypochlorite, Ethyl Alcohol, Activated Charcoal, Darkness and Benomyl. This experiment was conducted to reduce fungal and bacterial contamination of both the internal and external tissues. This experiment combined all previous treatments that had successfully reduced contamination by any degree (0.525% sodium hypochlorite, 70% ethyl alcohol, activated charcoal, darkness, and a benomyl soak). Explants were collected from apical meristems dissected from terminal shoots of 'Mohawk' pecan. The media contained 1.0 mg/liter BA and 3.0 g/liter activated charcoal. Explants were given a one week dark period. The sterilization treatments consisted of a benomyl soak for 4 1/2 hours, 70% ethyl alcohol (8 minutes), and 0.525% sodium hypochlorite (15 minutes). Explant material was collected on December 15, 1982. This experiment consisted of 80 replications.

CHAPTER IV

RESULTS AND DISCUSSION

Phenolics

Experiment I - Polyvinylpolypyrrolidone, Activated Charcoal, and Darkness

When added to the media, both PVP and activated charcoal reduced the amount of apparent phenolic compounds released to a minimum. PVP was difficult to incorporate into the media and settled to the bottom of the culture tube. Although not significant, treatments not given a dark period showed a slightly higher amount of phenolic-like compounds released from the explant tissue. Of all treatments tested, explants subjected to a one-week dark period with activated charcoal incorporated into the media gave the best response, with no apparent phenolics released from the explant.

Experiment II - Liquid Media Soak, and Sterile, Distilled, Deionized Water Soak

The liquid media presoak and the sterile, distilled, deionized water presoak were both used in combination with

PVP, activated charcoal, and dark treatments. There were no significant differences between these treatments. All treatment combinations were successful in reducing apparent phenolics 100%.

Experiment III - Charcoal and Darkness

Treatments that omitted either activated charcoal from the media, or a dark treatment did not successfully reduce apparent phenolic compounds. All of these treatments had some apparent phenolics released from the explant tissue. The treatment without activated charcoal and a dark treatment released the greatest amount of phenolic-like compounds. When activated charcoal was incorporated into the media in combination with one week dark treatment, no apparent phenolic compounds were released from the explant into the culture media.

Sterilization

Experiment IV - Antibiotics

All treatments of various combinations of antibiotic soaks and antibiotics incorporated into the media using Gentamicin sulfate, Nystatin, Penicillin-G, and Stieptomycin sulfate, did not reduce contamination. Each of the 16 different combinations of treatments had both fungal and bacterial contaminants within their

replications. Of the 80 cultures tested, 100% were contaminated.

Experiment V - Benomyl Soaks

Subjecting explants to a presoak using a saturated benomyl solution proved to be a reliable method for the control of fungal contamination. Explants not given a benomyl soak yielded 42% of the cultures infected with fungal contaminants (Table I). All other treatments utilizing a benomyl soak (3 hour, 6 hour, 12 hour, or 24 hour) exhibited no fungal contamination of any of the cultures. Although all benomyl treatments were successful in ridding the explants of fungal contamination, explants subjected to the 12 or 24 hour soaks suppressed growth of the explant tissue (Table II). Explants given the shorter soak periods allowed for the growth of the explant tissue, with 12% of the explant cultures expanding using the 6 hour soak, and expansion of 50% of the explants given the 3 hour soak.

Experiment VI - Ultra Violet,
Benzalkonium Chloride, Ethyl Alcohol,
and Sodium Hypochlorite

Although the number of contaminated cultures was very high for any of the various treatment combinations, ranging from 75% to 100%, the percent of contaminated cultures was not significant for any one of the treatments (Table III).

TABLE I

THE INFLUENCE OF BENOMYL SOAKS ON EXPLANT FUNGAL CONTAMINATION

Soaking Time[z] (Hours)	Contamination (%)
0	42a[y]
3	0b
6	0b
12	0b
24	0b

[z]Saturated benomyl solution.

[y]Means within columns followed by the same letters are not significantly different at the 0.05 level as determined by Duncan's multiple range test.

TABLE II

THE INFLUENCE OF BENOMYL SOAKS ON EXPLANT GROWTH

Soak Treatment[z] (Hours)	Growth[y] (%)
0	-
3	50
6	12
12	0
24	0

[z]Saturated benomyl solution.

[y]Any degree of apex expansion was counted as growth. Explants were either counted as growing or not growing.

TABLE III

THE INFLUENCE OF SELECTED STERILIZATION TREATMENTS ON NECROSIS, GROWTH AND CONTAMINATION

Time of Exposure				Necrosis	Gro
70% Ethyl Alchol (Minutes)	0.525% Sodium Hypochlorite (Minutes)	Benzalkonium Chloride (Minutes)	Ultra Violet Light (Hours)	(Index)[z]	(Ind
3	-	-	-	1.12b[y]	5.0
-	10	-	-	2.50ab	5.0
-	15	-	-	1.00b	5.0
-	20	-	-	1.40ab	5.0
3	10	-	-	1.12b	5.0
3	-	-	2	2.87a	4.6
-	10	-	2	1.85ab	3.5
3	10	-	2	1.37ab	5.0
-	-	5	-	3.25a	5.0
3	10	5	-	1.37ab	5.0

[z]Necrosis index; 0=No necrosis
1=Tip of meristem necrotic
2=1/2 of meristem necrotic
3=All of meristem necrotic
4=Most of explant necrotic
5=Total necrosis, death of explant

[y]Growth Index; 0=De
1=Br
2=Ye
3=Gr
4=Sw
5=Sh

[x]Each means represents five observations; contaminated cultures v replicates.

[w]Means within columns followed by the same letters are not signif at the 0.05 level as determined by Duncan's multiple range test.

All but the treatment combining ten minutes sodium hypochlorite and two hours ultra violet light yielded good growth according to the index used. The lowest necrosis index ratings occurred with the treatments combining 3 minutes ethyl alcohol, 15 minutes sodium hypochlorite or the treatment combining 3 minutes ethyl alcohol and 10 minutes sodium hypochlorite. Although not significant, these 3 treatments also yielded the highest growth indices and the lowest rate of contamination.

Experiment VII - Sodium Hypochlorite, and Ethyl Alcohol

Treatments of 70% ethyl alcohol (5, 10 and 20 minutes) and 0.525% sodium hypochlorite (10, 20, and 30 minutes) were unsuccessful in reducing contamination of explant tissues. Of the 40 cultures tested, 90% were contaminated. Although contamination was not reduced significantly using the 10, 20 or 30 minute soaks in 0.525% sodium hypochlorite (NaOCl), explants did not have a high necrosis index rating (Table IV). There were no significant differences in growth between any of the 0.525% NaOCl treatments (Table V).

Treatments using 5, 10 and 20 minute soaks in 70% ethyl alcohol (ETOH) did not significantly reduce contamination but the explants did not exhibit a high degree of necrosis and some cultures had expansion of the apex tissue (Table IV). After ten days from the start of the cultures, explants subjected to a 20 minute

TABLE IV

INFLUENCE OF 0.525% SODIUM HYPOCHLORITE ON EXPLANT NECROSIS AND CONTAMINATION

Sterilization Time 0.525% sodium hypochlorite	Days from start of cultures		Contamination
	10	25	
(Minutes)	(Necrosis Index)[z]	(Necrosis Index)	(%)
10	.42[y]b[x]	1.42a	92a
20	.93a	.26b	73a
30	.40b	.60ab	100a

[z]Necrosis Index; 0=No necrosis
1=Tip of meristem necrotic
2=1/2 of meristem necrotic
3=All of meristem necrotic
4=Most of explant necrotic
5=Total necrosis, death of explant.

[y]Each mean represents five observations, contaminated cultures were counted as lost replicates.

[x]Means within columns followed by the same letter are not significantly different at the 0.05 level as determined by Duncan's multiple range test.

TABLE V

INFLUENCE OF 0.525% SODIUM HYPOCHLORITE ON EXPLANT GROWTH

Sterilization Time 0.525% Sodium Hypochlorite (Minutes)	Days from start of cultures 17 (Growth Index)[z]	25 (Growth Index)
10	2.85[y]a[x]	1.07a
20	2.00a	2.80a
30	2.30a	1.60a

[z]Growth Index; 0=Death of explant
1=Browning of explant
2=Yellowing of explant
3=Green apex
4=Swollen apex tissue
5=Shoot apex expansion

[y]Each mean represents five observations, contaminated cultures were counted as lost replicates.

[x]Means within columns followed by the same letters are not significantly different at the 0.05 level as determined by Duncan's multiple range test.

TABLE VI

INFLUENCE OF 70% ETHYL ALCOHOL ON EXPLANT NECROSIS AND CONTAMINATION

Sterilization Time 70% Ethyl Alcohol (Minutes)	Days from start of cultures 10 (Necrosis Index)	25 (Necrosis Index)	Contamination (%)
5	.33[y]b[x]	1.33a	93.3a
10	.66ab	.46a	86.6a
20	.90a	.30a	80.0a

[z]Necrosis Index; 0=No necrosis
1=Tip of meristem necrotic
2=1/2 of meristem necrotic
3=All of meristem necrotic
4=Most of explant necrotic
5=Total necrosis, death of explant

[y]Each mean represents five observations; contaminated cultures were counted as lost replicates.

[x]Means within columns followed by the same letter are not significantly different at the 0.05 level as determined by Duncan's multiple range test.

sterilization soak with 70% ETOH had a significantly higher amount of necrosis than explants given a 5 minute soak, but after 25 days from the start of the cultures, there was no significant difference between the 5, 10, or 20 minute soaks in 70% ETOH.

Explants subjected to the five minute soak in 70% ETOH showed a significantly greater amount of growth after 17 days than either the 10 or the 20 minute soaks, but again, after 25 days from the start of the cultures there was no difference in growth between any of the 70% ETOH treatments (Table VII).

Experiment VIII - Sodium Hypochlorite, and Ethyl Alcohol

This experiment demonstrated that the sterilization treatment of 40 minutes in 0.525% sodium hypochlorite (NaOCl) gave a significantly better reduction in contamination than either the 20 minute or the 30 minute soaks, although there was no difference between the 40 minute and the 25 minute soaks (Table VIII). Seventy percent ethyl alcohol (ETOH) was effective in controlling contamination using 10, 20, and 25 minute soaks, however, the 15 minute soak was contaminated in 20% of the cultures.

As the sterilization time was increased using 70% ETOH, there was also a marked increase in necrosis. There

TABLE VII

INFLUENCE OF 70% ETHYL ALCOHOL ON EXPLANT GROWTH

Sterilization Time 70% Ethyl Alcohol	Days from start of cultures	
	17	25
(Minutes)	(Growth Index)[z]	(Growth Index)
5	4.00[y]a[x]	2.20a
10	1.20b	1.26a
20	2.00b	2.60a

[z]Growth Index; 0=Death of explant
1=Browning of explant
2=Yellowing of explant
3=Green apex
4=Swollen apex tissue
5=Shoot apex expansion

[y]Each mean represents five observations; contaminated cultures were counted as lost replicates.

[x]Means within columns followed by the same letters are not significantly different at the 0.05 level as determined by Duncan's multiple range test.

TABLE VIII

CONTAMINATION CONTROL USING SEPARATE TREATMENTS OF 70% ETHYL ALCOHOL AND 0.525% SODIUM HYPOCHLORITE

Surface Sterilization Treatment	Time (Minutes)	Contamination (%)
Sodium Hypochlorite	20	60a[z]
	25	20ab
	30	60a
	40	0b
Ethyl Alcohol	10	0b
	15	20ab
	20	0b
	25	0b

[z]Means within columns followed by the same letters are not significantly different at the 0.05 level as determined by Duncan's multiple range test.

was no difference in necrosis for any of the treatments using 0.525% NaOCl (Table IX).

The number of cultures exhibiting explant expansion was decreased as the time period for 70% ETOH soaks was increased (Table X). Best growth occurred at the 10 minute ETOH soak, which was a significantly higher amount of growth than either the 20 minute or 25 minute soaks. There were no significant differences between growth indexes given for the 20, 25, 30, or 40 minute soaks in NaOCl, although growth ratings for the 25 and 40 minute soaks were slightly higher than those for the 20 and 30 minute soaks.

Experiment IX - Sodium Hypochlorite, and Ethyl Alcohol

This experiment was designed to study the influence of 0.525% sodium hypochlorite (NaOCl) and 70% ethyl alcohol (ETOH) on contamination, necrosis, and growth of explant tissues. All the treatment combinations successfully controlled fungal contamination following a 4 hour presoak in a saturated benomyl solution. Of the treatments utilizing 0.525% NaOCl, there were no differences between the 10, 15, 20, or 25 minute soaks for reducing bacterial contamination (Table XI). None of the explants displayed a high degree of necrosis, with .94 being the highest necrosis rating (using a 0-5 necrosis index rating). There was no significant difference in

TABLE IX

THE INFLUENCE OF 70% ETHYL ALCOHOL AND 0.525% SODIUM HYPOCHLORITE ON EXPLANT NECROSIS

Surface Sterilization Treatment	Time (Minutes)	Necrosis[z] (Index)
Ethyl Alcohol	10	0.60[y]c[x]
	15	0.75c
	20	2.20b
	25	3.80a
Sodium Hypochlorite	20	0.20c
	25	0.60c
	30	1.00c
	40	1.40c

[z]Necrosis Index; 0=No necrosis
1=Tip of meristem necrotic
2=1/2 of meristem necrotic
3=All of meristem necrotic
4=Most of explant necrotic
5=Total necrosis, death of explant.

[y]Each mean represents five observations; contaminated cultures were counted as lost replicates.

[x]Means within columns followed by the same letters are not significantly different at the 0.05 level as determined by Duncan's multiple range test.

TABLE X

THE INFLUENCE OF 70% ETHYL ALCOHOL AND 0.525% SODIUM HYPOCHLORITE ON EXPLANT GROWTH

Surface Sterilization Treatment	Time (Minutes)	Growth[z] (Index)
Ethyl Alcohol	10	4.20[y] a[x]
	15	3.00ab
	20	1.60b
	25	1.00b
Sodium Hypochlorite	20	2.66ab
	25	4.50a
	30	3.20ab
	40	5.00a

[z] Growth Index; 0=Death of explant
1=Browning of explant
2=Yellowing of explant
3=Green apex
4=Swollen apex tissue
5=Shoot apex expansion

[y] Each mean represents five observations; contaminated cultures were counted as lost replicates.

[x] Means within columns followed by the same letters are not significantly different at the 0.05 level as determined by Duncan's multiple range test.

TABLE XI

THE INFLUENCE OF 0.525% SODIUM HYPOCHLORITE ON EXPLANT NECROSIS

Sterilization Time 0.525% Sodium Hypochlorite (Minutes)	Days from start of cultures 13 (Necrosis Index)[z]	23 (Necrosis Index)	Contamination %
10	.40a[y]	.06a	20a
15	.63a	.18a	25a
20	.94a	.25a	40a
25	.57a	.20a	20a

[z]Necrosis index; 0=No necrosis
1=Tip of meristem necrotic
2=1/2 of meristem necrotic
3=All of meristem necrotic
4=Most of explant necrotic
5=Total necrosis, death of explant.

[y]Means within columns followed by the same letters are not significantly different at the 0.05 level as determined by Duncan's multiple range test.

necrosis between any of the sterilization time periods at either 13 or 23 days after the start of the cultures.

There were no significant differences in growth between the 10, 15, 20, or 25 minute soaks in 0.525% NaOCl after either 13, 23, or 55 days from the start of the cultures (Table XII). Although the majority of the explants had swelling or expansion of the apex tissue after 13 days in culture, growth steadily declined after 23 and 55 days from the start of culture.

Treatment combinations using 70% ETOH for 6 minutes had the highest amount of contamination (Table XIII). The best control of contamination was with the 8 and 10 minute soaks in 70% ETOH, giving only 15 and 5% contamination, respectively. Although both the 8 minute and the 10 minute soaks reduced contamination, the 8 minute soak allowed for the best growth response after explants had been in culture for 55 days (Table XIV).

Experiment X - Sodium Hypochlorite, Ethyl Alcohol, Activated Charcoal, Darkness, and Benomyl

This experiment was important in that it demonstrated that even when explant tissue is collected in mid December, it is possible to get 60% uncontaminated cultures by using a 15 minute 0.525% sodium hypochlorite soak, 8 minute 70% ethyl alcohol soak, and a 4 1/2 hour benomyl presoak. This method was successful in reducing

TABLE XII

THE INFLUENCE OF 0.525% SODIUM HYPOCHLORITE ON EXPLANT GROWTH

Sterilization Time 0.525% Sodium Hypochlorite (Minutes)	Days from start of cultures		
	13	23	55
	(Growth Index)[z]	(Growth Index)	(Growth Index)
10	3.58[y] a[x]	2.80a	2.00a
15	3.71a	2.78a	1.94a
20	3.69a	2.58a	0.93a
25	3.66a	2.73a	1.42a

[z] Growth Index; 0=Death of explant
1=Browning of explant
2=Yellowing of explant
3=Green apex
4=Swollen apex tissue
5=Shoot apex expansion.

[y] Each mean represents five observations; contaminated cultures were counted as lost replicates.

[x] Means within columns followed by the same letters are not significantly different at the 0.05 level as determined by Duncan's multiple range test.

TABLE XIII

THE INFLUENCE OF 70% ETHYL ALCOHOL ON EXPLANT NECROSIS AND CONTAMINATION

Sterilization Time 70% Ethyl Alcohol	Days from start of cultures		Contamination
	13	23	
(Minutes)	(Necrosis Index)[z]	(Necrosis Index)	(%)
4	0.27b[y]	0.10a	25b
6	0.45b	0.21a	60a
8	0.63b	0.00a	15b
10	1.15a	0.42a	5b

[z]Necrosis Index; 0=No necrosis
1=Tip of meristem necrotic
2=1/2 of meristem necrotic
3=All of meristem necrotic
4=Most of explant necrotic
5=Total necrosis, death of explant

[y]Means within columns followed by the same letters are not significantly different at the 0.05 level as determined by Duncan's multiple range test.

TABLE XIV

THE INFLUENCE OF 70% ETHYL ALCOHOL ON EXPLANT GROWTH

Sterilization Time 70% Ethyl Alcohol	Days from start of cultures		
	13	23	55
(Minutes)	(Growth Index)[z]	(Growth Index)	(Growth Index)
4	3.88[y]a[x]	2.94a	1.37ab
6	3.82a	2.83a	1.70ab
8	3.53a	2.94a	2.31a
10	3.11a	2.21a	0.94b

[z]Growth Index; 0=Death of explant
1=Browning of explant
2=Yellowing of explant
3=Green apex
4=Swollen apex tissue
5=Shoot apex expansion

[y]Each mean represents five observations; contaminated cultures were counted as lost replicates.

[x]Means within columns followed by the same letters are not significantly different at the 0.05 level as determined by Duncan's multiple range test.

contamination to 17.5% after 27 days from the start of the cultures, and the highest contamination of 40% after 83 days from the start of culture.

CHAPTER V

SUMMARY AND CONCLUSIONS

Although methods have been established for the successful micropropagation of pecan, using juvenile tissue (15), micropropagation of pecan using adult tissue appears to be more difficult. One reason for the difficulty of micropropagation adult pecan-tissue is the inherent contamination of the internal tissues from field grown trees (20). Another problem with using adult tissues is that apparent phenolic compounds are released from the explant tissue into the media surrounding the tissue. These phenolics may become toxic to the explant within a matter of days.

Experiments conducted to reduce phenolics indicated that although PVP was successful in reducing phenolics,it was very difficult to incorporate the PVP into the media without it settling to the bottom of the culture tubes. The liquid media presoak and the sterile, distilled, deionized water presoak, both used in combination with PVP, activated charcoal, and dark treatments, did eliminate all apparent phenolic compounds released from the explant tissue, but these methods were very time

consuming and did not reduce phenolics any better than treatments utilizing activated charcoal and darkness. A treatment of three g/liter of activated charcoal incorporated into the media and darkness for one week followed by exposure to light for the duration of the experiment was successful in reducing phenolics 100%.

Nystatin, Gentamicin sulfate, Penicillin-G, or Streptomycin sulfate produced no reduction of either bacterial or fungal contamination. Subjecting explants to a 3 hour presoak using a saturated benomyl solution tested to be the best treatment for reducing fungal contamination, giving 100% reduction in fungal contaminants and not inhibiting explant growth. When 70% ethyl alcohol or 0.525% sodium hypochlorite were used independently for sterilizing explant tissues, contamination was not significantly reduced. However, a treatment of 70% ethyl alcohol for 8 minutes followed by 15 minutes in 0.525% sodium hypochlorite used in combination with a 4 1/2 hour soak in a saturated Benlate solution was successful in reducing contamination to 17.5% after 27 days from the start of the cultures and a final contamination rate of 40% after 83 days from the start of culture. This experiment was important in establishing a sterilization technique, whereby, explant tissue could be gathered during mid-December, and still yield 60% uncontaminated cultures.

A SELECTED BIBLIOGRAPHY

1. Abbott, A. J. and E. Whiteley. 1976. Culture of Malus tissue in vitro. I. Multiplication of apple plants from isolated shoot apices. Sci. Hort. 4:183-189.

2. Anderson, W. C. 1978. Tissue culture propagation of rhododendrons. In Vitro. 14:334.

3. Brutsch, M. O., P. Allan, and B. N. Wolstenholme. 1977. The anatomy of adventitious root formation in adult-phase pecan (Carya illinoensis(Wang.) K. Koch) stem cuttings. Hort Res. 17:23-31.

4. Button, J. and J. Kochba. 1977. Tissue culture in the citrus industry, p. 70-92. In: J. Reinert and Y.P.S. Bajaj (eds.) Plant cell, tissue, and organ culture. Springer-Verlag, Berlin, Heidelberg, New York.

5. Carlson, P. S., H. H. Smith, and R. D. Dearing. 1972. Parasexual interspecific plant hybridization. Proc. Nat. Acad. Sci. USA. 69:2292-2294.

6. Chaturved, H. C., A. K. Sharma, and R. N. Prasad. 1978. Shoot apex culture of Boogainvillea glabra 'Magnifica'. HortScience. 13:36.

7. Cheng, T. Y. 1976. Vegetative propagation of western hemlock (Tsuga heterophylla) through tissue culture. Plant and Cell Physiol. 17:1347-1350.

8. Childers, N. F. 1978. Trend toward high-density plantings with dwarfed trees in deciduous orchards. Proc. Fla. State Hort. Soc. 91:34-36.

9. Conger, B. V. 1981. Introduction, p. 1-4. In: B. V. Conger (eds.). Cloning agricultural plants via in vitro techniques. CRC Press, Boca Raton, Florida.

10. Durzan, D. J. and S. M. Lopushanski. 1975. Propagation of american elm via cell suspension cultures. Can. J. For. Res. 5:273-277.

11. Gautheret, R. J. 1939. Sur la possibilite de realiser la culture indefinie des tissue de tubercules de carotte. C. R. Acad. Sci. (Paris) 266:770-775.

12. Gupta, P. K., A. F. Mascarenhas, and V. Jagannathan. 1981. Tissue culture of forest trees-Clonal propagation of mature trees of Eucalyptus Citriodora Hook, by tissue culture. Plant Sci. Lett. 20:195-201.

13. Gupta, P. K., A. L. Nadgir, A. F. Mascarenhas, and V. Jagannathan. 1980. Tissue culture of forest trees: clonal multiplication of Tectona grandis L. (Teak) by tissue culture. 17:259-268.

14. Haberlandt, G. 1902. Kulturversoche mit isolierten pflanzensellen. Sitzungsb. Akad. Wiss. Wein, Math. Natur. Kl. 111:69-72.

15. Hansen, K. C. and J. E. Lazarte. 1982. In Vitro propagation of pecan (Carya illinoensis). HortScience 17:487. (Abstr.)

16. James, D. J. and I. J. Thurbon. 1979. Rapid in vitro rooting of the apple rootstock M. 9. J. of Hort. Sci. 54:309-311.

17. Jones, O. P., and M. E. Hopgood. 1979. The successful propagation in vitro of two rootstocks of Prunus: the plum rootstock Pixy (P. insititia) and the cherry rootstock F 12/1 (P. avium). J. Hort.Sci. 54:63-66.

18. Jones, O. P., C. A. Pontikis, and M. E. Hopgood. 1979. Propagation in vitro of five apple scion cultivars. J. Hort. Sci. 54:155-158.

19. Kartha, K. K. 1982. Cryopreservation of germplasm using meristem and tissue culture, p. 139-161. In: D. T. Tomes, B. E. Ellis, P. M. Harney, K. J. Kasha, R. L. Peterson (eds.). Application of plant cell and tissue culture to agriculture and industry. University of Guelph, Canada.

20. Knox, C. A. and R. H. Smith. 1980. A scanning electron microscope study of healthy pecan tissues showing the presence or absence of internal fungal contamination. In Vitro. 16:651-654.

21. Knox, C. A. and R. H. Smith. 1981. Progress in tissue culture methods for production of 'Riverside' stocks. Pecan Quarterly. 15:27-34.

22. Krikorian, A. D. and D. L. Berquam. 1969. Plant cell and tissue cultures: the role of Haberlandt. Bot. Rev. 35:59-88.

23. Krzewski, A., M. Witomska, and D. F. Millikan. 1980. Propagation of Platanus x acerfocia from meristem tips. J. Arbor. 6:250-252.

24. Lane, D. W. 1978. Regeneration of apple plants from shoot meristem tips. Plant Sci. Lett. 13:281-285.

25. Lane, D. W. 1980. Test tube propagation of apple and pear. Can. Agr. 25:24-26.

26. McEachern, G. R. 1973. The influence of propagation techniques, the rest phenomenon, and juvenility of propagation of pecan, Carya illinoensis. Doctoral Thesis. Texas A&M University, College Station, Texas.

27. Morel, G. M. 1964. Tissue culture-a means of clonal propagation of orchids. Am. Orchid Soc. Bull. 33:473-478.

28. Morel, G. M. 1966. Clonal propagation of orchids by meristem cultures. Cymbidium Soc. News. 20:3-11.

29. Murashige, T. 1974. Plant propagation through tissue cultures. Ann. Rev. Plant Physiol. 25:135-166.

30. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant., 15:473-497.

31. Negueroles, J. and O. P. Jones. 1979. Production in vitro of rootstock/scion combinations of prunus cultivars. J. Hort. Sci. 54:279-281.

32. Nobecourt, P. 1939. Sur la prinnite et l'angumentation de volume des cultures de tissues vegetaux. C. R. Acad. Sci. (Paris) 130:1270-1271.

33. Power, J. B., S. E. Cummins, and E. C. Cocking. 1970. Fusion of isolated plant protoplasts. Nature. 225:1016-1018.

34. Quak, F. 1977. Meristem culture and virus-free plants, p. 598-600. In:J. Reinert and Y.P.S. Bajaj (eds.). Plant cell, tissue, and organ culture. Springer-Veilag, Beilin, Heidelberg, New York.

35. Reinert, R. A. and H. C. Mohr. 1967. Propagation of Cattleya by tissue culture of lateral bud meristems. Proc. Am. Soc. Hort. Sci. 91:664-671.

36. Rodriguez, R. 1982. In vitro propagation of Castanea sativa Mill. through meristem-tip culture. HortScience 17:888-889.

37. Rugini, E. and G. Fontanazza. 1981. In vitro propagation of 'Dolce Agogia' olive. HortScience 16:492-493.

38. Sagawa, Y. and T. Shoji. 1967. Clonal propagation of dendrobiums through shoot meristem culture. Amer. Orchid Soc. Bul. 36:856.

39. Skirvin, R. M. 1981. Fruit crops, p. 51. In: B.B. Conger (ed.). Cloning agricultural plants via in vitro techniques. CRC Press.

40. Skirvin, R. M. and M. C. Chu. 1977. Tissue culture may revolutionize the production of peach shoots. Illinois Res. 19:18-19.

41. Skirvin, R. M. and M. C. Chu. 1978. Tissue culture of peach shoot tips. HortScience 13:349. (Abstr.)

42. Skirvin, R. M., M. C. Chu, and H. Rukan. 1980. Rooting studies with Prunus spp. in vitro. HortScience 15: 415 (Abstr).

43. Skoog, F., C. O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Sym. Soc. Exp. Bio. 11:118-131.

44. Smith, H. H. and I. A. Mastrangelo-Hough. 1977. Genetic variability through cell fusion, p. 265-286. In: W. R. Sharp, P. O. Larsen, E. F. Paddock, and V. Raghavan (eds.), Plant cell and tissue culture principals and applications. Ohio State Univ., Columbus.

45. Smith, M. W. 1977. Shoot meristem and callus tissue culture of pecans (Carya illinoensis (Wang) K. Koch). Ph.D. Thesis. Texas A&M Univ., College Station.

46. Smith, M. W., and H. J. Chiu. 1980. Seasonal changes in the rooting of juvenile and adult pecan cuttings. HortScience 15:594-595.

47. Snir, I. and A. Erez. 1980, In vitro propagation of malling merton apple rootstocks. HortScience 15:597-598.

48. Sriskandarajah, S., M. G. Mullins, and Y. Nair. 1982. Induction of adventitious rooting in vitro in difficult-to-propagate cultivars of apple. Plant Sci. Letters. 24:1-9.

49. Steward, F. C. 1958. Growth and organized development of cultured cells. III Interpretations of the growth from free cells of carrot plant. Am. J. Bot. 45: 709-713.

50. Steward, F. C., M. O. Mapes, A. E. Kent, and R. D. Holsten. 1958. Growth and development of cultured plant cells--biochemical and morphogenetic studies with cells yield new evidence on their metabolism and totipotency. Science. 143:20-27.

51. Steward, F. C., M. O. Mapes, K. Mears. 1958. Growth and organized development of cultured cells. II Organization in cultures grown from freely suspended cells. Am. J. Bot. 45:705-708.

52. Steward, F. C., M. O. Mapes, and J. Smith. 1958. Growth and organized development of cultured cells. I Growth and division of freely suspended cells. Am. J. Bot. 45:693-703.

53. Stoutemyer, V. T. 1938. Root hardwood cuttings with acids. Am. Nurseryman. 68:3-5.

54. Street, H. E. 1973. Introduction, p. 1-10 In: H. E. Street (eds.). Plant tissue and cell culture. University of California Press, Berkeley.

55. Tabachnik, L. and D. E. Kester. 1977. Shoot culture for almond and almond-peach hybird clones in vitro. HortScience. 12:545-547.

56. Takebe, I. G. Labib, and G. Melchers. 1971. Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwissenschaften 58:318-320.

57. Vasil, V. and A. C. Hilderbrandt. 1965. Differentiation of tobacco plants from single, isolated cells in microcultures. Science. 150:889-892.

58. Werner, E. M. and A. A. Boe. 1980. In vitro propagation of Malling 7 apple rootstocks. HortScience 15:509-510.

59. White, P. R. 1934. Potentially unlimited growth of excised tomato root tips in a liquid medium. Plant Physiol. 9:585-600.

60. White, P. R. 1939. Potentially unlimited growth of excised plant callus in an artificial nutrient. Am. J. Bot. 26:59-64.

61. Wimber, D. E. 1963. Clonal multiplication of Cymbidiums through tissue culture of the shoot meristem. Am. Orchid Soc. Bul. 32:105-107.

62. Wood, B. W. 1982. In vitro proliferation of pecan shoots. HortScience 17:890-891.

63. Zenk, M. H. 1978. Introduction, p. 1. In: T. A. Thorpe (eds.). Frontiers of plant tissue culture. Univ. of Calgary Press, Calgary.

64. Zenk, M. H., H. El-Shagi, H. Arehs, J. Stockigt, E. W. Weiler, B. Deus. 1977. Secondary products, p. 27. In: Barz, E. Reinhard, M. H. Zenk (eds.). Plant tissue culture and its biotechnological application. Springer-Veilag, Berlin.

65. Zimmerman, R. H. and O. C. Broome. 1979. In vitro propagation of apple cultivars. HortScience 14:102.

VITA

Brenda Carol Cotten

Candidate for the Degree of

Master of Science

Thesis: MICROPROPAGATION OF PECAN

Major Field: Horticulture

Biographical:

Personal Data: Born in Jackson, Mississippi, September 21, 1959, the daughter of Howard M. and Bobbie F. Cotten.

Education: Graduated from Memorial High School, Tulsa, Oklahoma, in 1977; received the Bachelor of Science in Agriculture from Oklahoma State University in 1981 with a major in Horticulture, completed the requirements for the Master of Science degree in Horticulture at Oklahoma State University in July, 1983.

Professional Experience: Graduate Research Assistant, Horticulture, Oklahoma State University, 1981-1983.