MICROPROPAGATION OF BLACK WALNUT:

A STUDY OF VARIATION

IN VITRO

BY

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1987

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 1990

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ACKNOWLEDGMENTS

I wish to express sincere appreciation to Dr. Charles Tauer for his advice and financial support for this research project. Many thanks also go to Dr. Arron Guenzi and Dr. Becky Johnson for serving on my graduate committee. Their suggestions and support were very helpful throughout this study.

My parents, Dr. Peter H. Allen and Marion L. Allen, gave vital encouragement and allowed me the freedom to pursue my goals. I would also like to thank David Gunther, Gene Baker and George Surritte for their help during the course of this study.

Seed collections were received from Dr. Terry Robinson, James Zaczek, Dr. Harold Gerhold, Bill Haywood and Russell Cox. Their help was greatly appreciated.

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CHAPTER I

INTRODUCTION

Black walnut (Juglans nigra L.) is a highly valued timber and nut species. Nut meats are an important food crop for both human and wildlife consumption. Individual trees have sold for as much as $35,000 for lumber (Beineke, 1983). Black walnut wood is easily machined and has good dimensional stability and resistance to decay (Beineke, 1983). Its highly valued wood is used in veneer, furniture and other items where its characteristic luster and quality are needed.

The natural range of black walnut includes most of eastern and central North America, from New Hampshire south to Georgia and west to Texas and South Dakota. Its prime range includes Illinois, Indiana, Iowa, Kentucky, Missouri and Ohio, but it is also economically important in other states, including Oklahoma.

As a species, black walnut has been subjected to high-grading or dysgenic selection since early in the history of the United States. The resulting reduction in quality but continued demand has led to the establishment of genetic improvement programs. J. nigra is usually propagated from sexually produced seedlings for use in commercial

plantations (McGranahan et al., 1987). Unfortunately, seed production is not heavy or reliable, particularly in the case of high quality timber trees (Funk, 1970). In dense stands, black walnut nut production can be severely limited. Nut production is cyclic in most trees and large quantities of nuts may be produced every 2-4 years, or may never be produced. For reproduction purposes, select trees in natural populations often must be propagated vegetatively.

Black walnut has been identified as one of the most difficult tree species to propagate vegetatively using conventional methods (McKay, 1966). Attempts to root black walnut cuttings have not led to routine or consistent success (Beineke, 1983). The success of grafting is dependent on a number of factors including the age of the donor tree, scion storage methods, rootstock condition, grafting technique and graft aftercare. Beineke (1983) reported a success rate of up to 70% under ideal conditions. Preliminary trials in our greenhouse have resulted in 20-30% success with scion material from mature select trees from wild populations in Oklahoma. The rate of multiplication by grafting a superior genotype is limited by the number of scions collected, growth rate and the graft success rate.

In vitro micropropagation of black walnut would allow a dramatic increase in the rate and number of superior individuals one can produce from a single select phenotype. Vegetative propagation and establishment of clonal

plantations could potentially double short term genetic gains (Zobel, 1981). When regeneration by seed is used, only the additive portion of the genetic superiority (narrow sense heritability) is captured while vegetative propagation allows the capture of all the genetic potential (broad sense heritability), including dominance and interactive effects.

Beineke (1983) stated that successful micropropagation from mature tissue would allow rapid multiplication of improved cultivars from a few buds and will probably be cheaper than grafting. Stefan and Millikan (1985) reported black walnut shoot multiplication rates in vitro of 10 fold in 2 months and 300 fold in 5 months. However, they were unable to root the micropropagated shoots produced from mature tree explants (a very small cutting from the donor plant introduced in vitro). The high value of mature black walnut trees should compensate for the possible increased costs of micropropagation techniques compared to conventional breeding methods. The costs of micropropagated plantlets should become economically competitive at high production rates.

The ability to regenerate trees from single cells would allow the application of genetic manipulation techniques as a tree improvement tool. McGranahan et al. (1988) demonstrated that agrobacterium-mediated transformation of English walnut (Juglans regia L.) is possible using somatic embryos. Only four transgenic plantlets were rooted,

demonstrating the limitations of present techniques. These techniques must be applied to tissue from mature trees before their full potential can be realized. The applications of these methods should have a profound effect on the improvement of walnut species. A reliable tissue culture program is one prerequisite of any genetic transformation system.

Many plant species, including black walnut, have shown intraspecific variation in response in vitro for both shoot and root growth (Heile-Sudholt et al., 1986 and Jay-Allemand et al., 1988B). Bonga and Durzan (1982) stated that inter- and intra-specific genetic variation in regeneration response is a common problem with most tree species. They reported that one frequently encounters wide spread genotypic variation within half-sib and full-sib families in the micropropagation of trees.

The objective of this study was to examine geographic variation in the micropropagation of black walnut. Intraspecific variation in growth rate and differentiation was examined by micropropagation of seedlings from a number of geographic areas within black walnut's natural range. The first step of this study was to refine the techniques for the multiplication and regeneration of juvenile material (seedlings), using local plant material. The methods used in previous studies where seedling materials were used with black walnut and closely related species (McGranahan et al.,

1987, Heile-Sudholt et al., 1986, Stefan and Millikan, 1983, 1985 and 1986, Driver and Kuniyuki, 1984 and Somers et al., 1982) were evaluated.

CHAPTER II

LITERATURE REVIEW

Introduction

The study of micropropagation of walnut (Juglans sp.) began in the late 1960's (Cummins and Ashby, 1969) but it wasn't until the 1980's that intensive study began (Rodriguez, 1982A and 1982B, Stefan and Millikan, 1983, 1984, 1985 and 1986, Tulecke and McGranahan, 1985, Heile-Sudholt, 1986 and McGranahan et al., 1987). Stefan and Millikan (1983, 1985 and 1986), Heile-Sudholt (1986) and McGranahan et al. (1987) worked with tissue from mature walnut trees. McGranahan et al. (1988) utilized tissue culture methods in the genetic transformation of somatic embryos of English walnut (Juglans regia L.) using Agrobacterium tumefaciens.

Every phase of the culture cycle, including the ingredients of the media, the genotype, the cultural environment, and the condition of the donor plant have been shown to affect walnut's ability to grow and differentiate in culture.

Explant Source

Physiologic Age and Condition of the Donor Plant

In general, the specific plant tissue used as an explant source has a direct effect on growth and differentiation *in vitro*. This is especially true for black walnut. The most common source of plant cells for callus and suspension culture of trees is the cambial zone (Dodd, 1983). Cambial tissue is undergoing high levels of mitotic activity and has a greater ability to grow and differentiate in culture. The genotype of the individual and the tissue's location on the plant effect its ability to be cultured.

The physiological age of the explant tissue influences the type and extent of morphogenesis in culture. Younger, less differentiated tissues generally have greater morphogenic potential. Explants from mature 'Paradox' walnut (a *J. hindsii* X *J. regia* hybrid) trees were found to be more difficult to culture due to excessive callusing, slow growth and contamination than juvenile tissue (McGranahan et al., 1987).

Cotton (1983) attempted to solve internal contamination problems associated with the micropropagation of mature Pecan (*Carya illinoensis*) tissue. She reported that the age of the donor plant seemed to have a direct effect on the level of internal contamination in culture and that

tissue from older plants had higher contamination levels.

A number of different types and ages of donor tissue have been used in studies of the micropropagation of Juglans. Cummins and Ashby (1969) used tissue from the present year's growth from two year old seedlings but were unable to progress beyond callus formation. They stripped the bark off 10 cm segments of one year old black walnut whips. They then cut triangular wedges of cambial tissue (~1 cm on a side), for use as explants. Callus growth was observed with no organogenesis visible.

Somers et al. (1982) used greenhouse reared black walnut seedlings as a source of juvenile tissue but were unable to root micropropagated shoots.

Stefan and Millikan (1983) cultured meristems from mature J. nigra trees. Initially, they were unable to sustain callus growth beyond 8 weeks. In a later attempt Stefan and Millikan (1985) produced 6 generations of subcultured plantlets but were unable to stimulate root growth using a variety of methods. Stefan (1989) reported that micropropagated shoots were grafted to seedling rootstocks. Three cuttings from these grafts were successfully rooted. Meynier (1985) isolated meristems from a hybrid walnut (J. nigra L. X J. regia L.) to produce rooted plants. Explants were derived from a clone cut back annually.

Heile-Sudholt et al. (1986) used 20-30 cm tall black

walnut seedlings and successfully micropropagated rooted plantlets. They also attempted to culture shoot tips from mature black walnut trees but internal contamination prevented successful establishment in vitro. McGranahan et al. (1987) used stump sprouts and 1 to 3 month old greenhouse and field grown 'Paradox' walnut (J. hindsii x J. regia) seedlings to successfully produce micropropagated plants.

Rodriguez and Sanchez-Tames (1981) reported the first morphogenesis in walnut tissue culture using stem segments, leaf disks, whole peeled cotyledons and root segments of J. regia. Roots were obtained from callus derived from cotyledons and root segments. They concluded that stem segments were non-morphogenic while cotyledons and roots were morphogenic.

Caruso (1983) removed the embryos from stratified black walnut seeds for use as a tissue source to produce rooted shoots. Chalupa (1981) succeeded using nodal segments from J. regia (Persian or English walnut) seedlings to produce rooted plantlets. Tulecke and McGranahan (1985) achieved somatic embryogenesis from mature seeds of J. regia. After a cold treatment of 8-10 weeks somatic embryos grew into complete plants. Cornu (1988A and 1988B) extracted the whole embryo with cotyledons from mature J. nigra seeds to produce somatic embryos. A small fraction of these somatic embryos grew to complete plants.

Polito et al. (1989) produced somatic embryos from embryogenic cultures of Juglans regia and determined that somatic embryos arise from single cells. This was an important discovery in that a genetically transformed individual must develop from a single transformed cell.

Physiologic State

Another important factor, the time of year the explant is isolated, may affect explant growth, the extent of internal contamination, and explant morphogenic potential. Jay-Allemand et al. (1988A) reported significant variation among 21 different phenolic compounds during growth and dormancy phases of hybrid walnut (J. nigra L. X J. regia L.) using high performance liquid chromatography (HPLC) analysis. Data were analyzed using canonical discriminant analysis on HPLC peak area and peak area ratios. They found that three ratios of five polyphenols were related to the rejuvenation phenomenon. Seedlings and stump sprouts showed similar levels of juvenility and were easily discriminated from shoots from mature trees. Jay-Allemand et al. (1988A) noted that quantitative variation of polyphenols is influenced by genotype, cultural treatment, physiological factors (relations between roots and shoots) and the type of plant tissue. They report that polyphenols are well known as physiological indicators of plant development.

Jay-Allemand et al. (1988B) found clonal variation in

multiplication and rooting rate was linked to polyphenolic content of *in vitro* hybrid walnut tissue (*J. nigra* X *J. regia*).

Dodd (1983) stated that the concentration of growth regulators in trees, particularly hardwoods, shows seasonal variation and specifically that spring cambial explants of trees were suitable for tissue culture purposes due to their ability to undergo a rapid increase in the rate of cell division. Cummins and Ashby (1969) isolated their explants in March in their unsuccessful work with black walnut seedlings. They found high levels of contamination (27/40 jars) with a *Rhizopus* mold that appeared to originate from the pith of the explant. Revilla et al. (1988) noted that *in vitro* response (growth and differentiation) of axillary buds of *J. regia* shows seasonal variation.

Stefan (1989) gathered shoot tips from mature black walnut at the end of June, when the annual flush of growth was complete and the terminal buds were set, to minimize microorganism levels. The microshoots produced have resisted rooting procedures but have been successfully grafted to seedling root stocks. Three cuttings from micropropagated shoot grafts have been rooted by dipping cuttings in 3% IBA and placing them in a mist chamber.

Pre-Treatment

Pre-treatment of black walnut explant tissues may

improve survival and differentiation in culture. Stefan and Millikan (1985) sealed the cut ends of shoots with paraffin for storage in plastic bags until they were used as explants. Six-centimeter long shoot segments were soaked overnight in 0.01 M sodium diethyldithiocarbamate (Na DIECA) to prevent oxidation of phenols prior to surface sterilization. This procedure was followed to prevent lethal browning. Explants survived and grew to produce micropropagated shoots.

The rejuvenation of tissue from mature trees before explant isolation may allow greater morphogenesis in culture. McGranahan and Driver (1987) reported that rejuvenation of 'Paradox' walnut tissue from mature trees could be achieved in the following manner. Dormant scion wood from selected trees was grafted onto seedling root stocks. After the first flush in the spring had grown 30 to 60 cm, it was cut back to 3-4 buds. The remaining buds on cut back branches were sprayed every 3-4 days with a solution of benzylaminopurine (BAP, 100 mg/L) and gibberellic acid (GA_3, 50 mg/L) to induce vigorous growth. Shoots were cut back to 3-4 buds every time they reach 15 to 20 cm in length. Tauer (personal communication, 1990) applied the regeneration technique described above to grafts from mature black walnut trees. When grafted shoots were cut back, they did not grow vigorously and the procedure could not be repeated during a growing season.

Fungicides may be used to control internal contamination of the donor plant prior to explant isolation. Revilla et al. (1988) grew J. regia L. plants in a greenhouse where phytosanitary treatments were applied for 3-6 months before collection of explants. They reported that phytosanitary treatments and surface sterilization did not eliminate endogenous bacteria, prompting their proposal to study alternative surface sterilization methods and the inclusion of antibiotics in the culture media.

Explant Surface Sterilization

The methods used to surface sterilize plant tissue prior to establishment in vitro appears to be an important factor affecting tissue culture success. Cummins and Ashby (1969) scrubbed explants from two year old black walnut seedlings for 10 minutes in 1.5% sodium hypochlorite (NaOCl) followed by rinses in 50% ethanol and sterile water, but had severe contamination problems using this procedure. Somers et al. (1982) and Heile-Sudholt et al. (1986) surface sterilized black walnut explants from seedlings in a 15 minute soak in 0.5% NaOCl and 0.01% Tween 20 detergent followed by three five minute rinses in sterilized distilled water. They found that it was impossible to remove all microorganisms using these methods. Aseptically removed embryos did not require surface sterilization (Somers et al., 1982).

Ettinger and Preece (1985) also used the surface sterilization technique described by Somers et al. (1982) with Rhododendron hybrids. They did not report contamination problems. McGranahan et al. (1987) used a similar technique with 1% NaOCl (20% commercial bleach) with 'Paradox' walnut. Latent contamination was reported in tissue from mature trees. Revilla et al. (1988) noted that one of the major problems faced in the micropropagation of J. regia is that superficial surface sterilization of the material does not eliminate endogenous bacteria.

Cotton (1983) examined the use of antibiotic (Gentamicin sulfate 100 mg/l and Nystatin 100 mg/l) soaks to control bacterial and fungal contamination in the micropropagation of pecan and found them ineffective. She reported that explants soaked in benomyl (saturated solution) up to 24 hours prior to establishment in culture provided reliable control of fungal contamination but that soaks over 12 hours suppressed growth.

Stefan (1989) reported that shoot tips of black walnut were often infected with Botrytis (a genus of imperfect fungi), and recommended a 2 minute soak in 70% ethanol followed by a 20 minute soak in 30% clorox to effectively surface sterilize explants prior to establishment in vitro.

Media

Types of Media Used

The type of media used to culture walnut affects the degree of explant growth and differentiation. Somers et al. (1982) compared MS (Murashige and Skoog, 1962) medium, WP (Woody Plant) medium (Lloyd and McCown, 1981), VV medium (Vieitez and Vieitez, 1980) and WB medium (White, 1940) for black walnut. They reported that black walnut explants on WP and MS media turned green and grew faster then on VV and WB media.

Driver and Kuniyuki (1984) tested media components individually for use with gelrite (gellum gum, a gelling agent) to form Driver and Kuniyuki walnut (DKW) media, specific for 'Paradox' walnut. They reported that their DKW media supported increased growth rates with reduced tendency for callus formation after repeated subculturing when compared with WP, B5 (Gamborg et al., 1968), MS (Murashige, 1974) and Cheng's (Cheng, 1978) media. Driver and Kuniyuki (1984) found that explants performed better on WP and B5 than on MS or Cheng media.

McGranahan et al. (1987) used a corrected version of DKW media in which errors in the original published DKW media protocol (Driver and Kuniyuki, 1984) were corrected to the actual formula (DKW-C) (APPENDIX A). They regenerated plants from 'Paradox' walnut, northern California black

walnut (J. hindsii) and Pterocarya stenoptera C. DC. (a chinese Wing-nut). DKW-C contains a reduced concentration of myo-inositol (0.55 mM) and K_2SO_4 (8.9 mM) in place of KH_2SO_4 compared to the original published version of DKW media.

Stefan and Millikan (1986) used a modified liquid DKW (mDKW) media for the first 21 days with black walnut meristems which contained reduced sucrose (15 g/liter), 2% polyvinylpyrrolidone (PVP) and no copper. After the first 21 days explants were transferred to solid mDKW media solidified with agar. They rapidly multiplied micropropagated shoots but were unable to stimulate root growth. Stefan and Millikan (1986) noted that 2.0% coconut milk supported longer life for the micropropagated shoots.

Heile-Sudholt et al. (1986) compared WP and a modified DKW (30 g/l sucrose) media for use with black walnut and found that cultures on DKW media produced more axillary shoots, thinner microshoots, had greater leaf expansion and were greener.

Phenolic Production

A number of methods have been used to prevent harmful levels of explant exudate (phenolics) build-up in the media. PVP has been added to media to absorb exudate from black walnut. Somers et al. (1982) found that PVP did not absorb exudates and actually may have promoted explant necrosis.

In contrast, Stefan and Millikan (1984) found that the addition of PVP and sodium diethyldithiocarbamate, (Na-DIECA), reduced enzyme activity (oxidation of phenolic compounds) and lethal browning in black walnut. They also recommended that the media sucrose level be reduced by 50% from 50 g/l to 15 g/l. Somers et al. (1982) recommended the employment of a rapid transfer technique (transfers on days 1, 3, and 5) to solve problems with black walnut explant necrosis. Stefan and Millikan (1986) used 2.0% PVP in liquid mDKW media for 21 days before transferring their explants to solid mDKW media to produce healthy in vitro black walnut shoots.

Heile-Sudholt et al. (1986) successfully regenerated black walnut by initially transferring explants to new media on days 1, 3 and 5 followed by biweekly transfers. McGranahan et al. (1987) recommended that 'Paradox' walnut, northern California black walnut and Pterocarya stenoptera explants be transferred to fresh media when it appeared discolored and that 2% walnut endosperm extract improved establishment and growth. McGranahan et al. (1987) concluded that transfers may be required daily for the first week and then weekly until the growing bud is excised from the nodal explant. Biweekly transfers were advised thereafter.

Ettinger and Preece (1985) determined that the dark exudate in their medium was not related to explant survival

in their work with rhododendron hybrids. Cotton (1983) found that PVP (1 mg/l), a one-week dark treatment and activated charcoal (3.0 g/l) reduced the amount of apparent phenolic compounds released in the media by pecan explants. A treatment of 3 g/l activated charcoal in the medium, with darkness for the first week, reduced phenolics 100%.

Plant Growth Regulators

Plant growth regulators control growth and differentiation of tissue in culture. The ratio of auxin to cytokinin seems to determine the type and extent of organogenesis. Dodd (1983) stated that the ability to regenerate complete plants is dramatically affected by the concentration of plant growth regulators, specifically the auxin to cytokinin ratio. Plant hormones are usually included in culture media to keep plant tissue viable and stimulate cell division, cell expansion and morphogenesis.

Auxins are used to induce and maintain callus tissue in culture. Auxins cause cell elongation, swelling of tissues, and cell division (callus formation), and inhibits adventitious shoot formation in culture. Cytokinins promote cell division and adventitious shoot production while inhibiting root formation. Adventitious shoots are promoted by decreasing apical dominance (Pierik, 1987). Gibberellins affect cell elongation as well as flowering processes and seed germination. Bonga and Durzan (1982) concluded that

gibberellins were not important in morphogenic processes or that endogenous levels are sufficient in the tissues of most tree species.

Interactions between plant growth regulators complicate their application to tissue culture. Pierik (1987) noted that plant growth regulators interact with a number of factors in the culture cycle. Gibberellins are very heat sensitive, up 90% of their biological activity is lost during autoclaving (Pierik, 1987). Studies which did not take this into account should be interpreted with caution. Endogenous levels of auxin or cytokinins are strongly affected by temperature. Indoleacetic acid (IAA) is broken down more rapidly in light than in dark. In addition, different genotypes may require different concentrations of growth regulators for growth and development.

Cytokinin biosynthesis may be activated by high levels of auxin and vice versa (Bonga and Durzan, 1982). Manmade analogs of growth regulators were often more effective than the endogenous compounds in promoting growth and differentiation in culture.

Somers et al. (1982) reported that 0.9 to 4.4 uM (0.2 to 1.0 mg/l) BAP (N^6-Benzylaminopurine, a cytokinin) in combination with 0.0 to 0.5 uM (0.0 to 0.1 mg/l) IBA (indole-3-butyric acid, an auxin) approach optimum conditions for promotion of axillary shoot growth of juvenile black walnut explants. They also reported that GA_3

(gibberellic acid, a gibberellin) had no effects on shoot proliferation or elongation but did increase callus growth.

Caruso (1983) reported that the development of axillary shoots from mature black walnut embryos was optimal when 8.9 uM (2 mg/l) BAP and 11.4 uM (2 mg/l) indoleacetylphenylalanine (IAAPhe, an auxin) were included in the media, compared to each growth regulator individually. Driver and Kuniyuki (1984) found that 4.5 uM (1.0 mg/l) BAP and 5 nM (.001 mg/l) IBA were optimum for multiple shoot development on WP medium with 'Paradox' walnut.

Heile-Sudholt et al. (1986) concluded that axillary shoot proliferation of black walnut required BAP, with 10 uM (2.3 mg/l) being the optimum level, but that BAP inhibited elongation of the radical. Stefan and Millikan (1985) included 17.8 uM (4.0 mg/l) BAP, 9.8 uM (2.0 mg/l) 2iP (isopentenyladenine, a cytokinin), 0.5 uM (0.1 mg/l) NAA (naphthaleneacetic acid, a synthetic auxin) and 2.9 pM (0.001 ug/l) GA_3 in mDKW media to obtain six generations of subcultured plantlets from mature tree tissue.

Ettinger and Preece (1985) concluded that 2iP was necessary for explant growth and survival of rhododendron P.J.M. hybrids. Cummins and Ashby (1969) observed that black walnut callus tissue grew most vigorously with 26.8 uM (5 mg/l) NAA and 0.9 uM (0.2 mg/l) kinetin in the media.

Antibiotics

The inclusion of antibiotics in the culture media has been suggested as a solution to internal contamination problems *in vitro*. The addition of antibiotics to the media in high enough concentrations to control contaminants may inhibit growth and differentiation of higher plants (Pierik, 1987). Antibiotics, in general, are not heat stable and should be filter sterilized for inclusion in culture media (Pierik, 1987).

Young et al. (1984) examined the effectiveness of gentamicin sulfate, amikacin sulfate, tetracycline hydrochloride, polymyxin B sulfate, rifampicin, carbenicillin-disodium salt and cefotaxime-sodium salt at concentrations 6 to 100 mg/l in eliminating bacterial contamination and limiting phytotoxic effects. They found that no single antibiotic was effective against bacterial contaminants in shoot culture of woody plants but that a combination of different antibiotics was. Young et al. (1984) concluded that bacteria could be eliminated with low levels (6-25 mg/l) of cefotaxime, tetracycline, rifampicin and polymyxin B with no deleterious effects on the shoots. The species examined included apple (*Malus*), walnut (*Juglans*), pear (*Pyrus*), birch (*Betula*), filbert (*Corylus*), Douglas-fir (*Pseudotsuga menziesii*), mountain laurel (*Kalmia*) and rhododendron (*Rhododendron*). All antibiotics were filter sterilized before being added to autoclaved

media.

Faulkner et al. (1974) used a combination of ampicillin (250 mg), furazolidone (100 mg), oxytetracycline hydrochloride (250 mg), streptomycin sulfate (250 mg) and captan (250 mg) to effectively disinfest both alfalfa seeds and stem nematodes. Cotton (1983) experimented with the inclusion of a number of antibiotics (gentamicin sulfate 50 mg/l, nystatin 50 mg/l, penicillin-G 62.5 mg/l and streptomycin 100 mg/l) in the culture media to control bacterial and fungal contamination in the micropropagation of pecan. Cotton (1983) examined the antibiotics both singly and in combinations of twos to determine their effectiveness in eliminating contamination, but no combination proved effective. Cotton (1983) reported 100% contamination of the explants; however, antibiotics appear to have been added to the media before autoclaving. This may result in the degradation of the antibiotics and eliminate their effectiveness (Pierik, 1987).

Revilla et al. (1988) noted that superficial sterilization of J. regia explant tissue does not eliminate endogenous bacteria. They reported that the addition of some antibiotic mixtures to the culture media allowed the recovery of a high percentage of explants that were contaminated with bacteria. Driver (personal communication, 1989) examined the use of a number of antibiotics in the culture media of 'Paradox' walnut without success. Again,

all antibiotics were autoclaved.

Gelling Agent

Two methods have been used to solidify media in the micropropagation of walnut. McGranahan et al. (1987) used Gelrite (trademark of Merck, U.S.A., a division of Kelco) in DKW-C to produce a clear solid medium for 'Paradox' walnut. Gelrite, a gellum gum, is a natural anionic heteropolysaccharide that solidifies a media after a heating cycle in the presence of soluble salts (Pierik, 1987). DKW media was designed as a 'Paradox' walnut specific medium with Gelrite as the solidifying agent.

Another gelling agent, agar, is a highly purified seaweed derivative which is a polysaccharide with high molecular mass. It forms a gel that binds with water and adsorbs compounds. Pierik (1987) noted that agar contains organic and inorganic contaminants (ash, calcium, silica, chloride). Heile-Sudholt et al. (1986) used agar in a modified DKW media which may have altered the concentration of available vitamins and minerals in the media from the optimum as determined by Driver and Kuniyuki (1984). Difco Bacto agar was used by Ettinger and Preece (1985) and Somers et al. (1982). Ettinger and Preece (1985) observed that rhododendron shoot tips in stationary liquid cultures did not survive while those on solid media did. Rapid transfer techniques did not solve this problem.

Myo-inositol

Myo-inositol, a component of coconut liquid endosperm, has been observed to promote growth and differentiation of tissue in culture. In higher plants it is involved in the synthesis of phospholipids, cell wall pectins and cytoplasmic membranes. Stefan and Millikan (1986) stated that coconut milk appeared to contain substances that promoted longer life in micropropagated black walnut shoots. DKW-C media contains 100 mg/l of myo-inositol.

Cultural Environment

Light intensity, wave length and duration are important factors for inducing organogenesis in tissue culture. Pierik (1987) reported that irradiance levels higher than 8-15 watts/meter^2 (W m^{-2}) may be advantageous to woody species. Photosynthesis should not be necessary for tissue growth in culture because a carbon source is available in the media in the form of sucrose. Light has been reported to inhibit root growth in some species.

Somers et al. (1982) used 5 W m^{-2} of photosynthetically active radiation (PAR) from cool white fluorescent lights for 16 hours per day to generate black walnut microshoots *in vitro*. Heile-Sudholt et al. (1986) used similar environmental conditions with 4.9 W m^{-2} PAR to produce rooted plantlets with black walnut. Driver and Kuniyuki (1984) kept paradox walnut cultures in constant

light from cool white fluorescent lamps to successfully produce rooted shoots . Cummins and Ashby (1969) incubated their black walnut cultures in the dark and were unable to stimulate organogenesis.

Rooting of Microshoots

One of the final steps in the tissue culture of walnut involves the generation of roots on micropropagated shoots. This may be achieved in vitro or by transferring microshoots from solid media to soil or vermiculite. Preliminary results suggest that genotype and juvenility influence 'Paradox" and black walnut rooting (McGranahan et al., 1987 and Jay-Allemand et al., 1988B).

McGranahan et al. (1987) recommended several changes in the cultural environment be implemented to stimulate root growth of 'Paradox' walnut, with a two stage rooting procedure. The rooting procedure involved increasing the light intensity from 50 to 66 uE m^{-2} s^{-1}, (a unit of irradiance), shortening the photoperiod from 24 to 17 hours and reducing the temperature from 28 to 19 degrees Celsius. Vigorous shoots were pretreated by cutting the basal end and placing them on DKW-C rooting media containing adjusted levels of sucrose (increased from 30 g/l to 53 g/l), $Ca(NO_3)*4H_2O$ (634.0 mg/l), NH_4NO_3 (456.2 mg/l) and IBA (0.15 mg/l) for 7 days followed by a wash in a slurry of systemic fungicides (Benalate, Bravo and Tersan, 1 tsp/l), and a dip

in rooting powder (2 g IBA and 100 g talc). McGranahan et al. (1987) reported that initial root growth is five times more rapid than shoot growth in micropropagated plantlets, making transplanting difficult by conventional means and potting container size a critical factor. They recommended that plantlets be placed directly in the field when the base of the shoot begins to swell.

Jay-Allemand et al. (1988B) determined that clonal variability in rooting rates was linked to polyphenolic content in hybrid walnut (J. nigra X J. regia). Driver and Kuniyuki (1984) found that 30 uM IBA resulted in 70% of the shoots forming roots with 'Paradox' walnut on WP media in vitro. They also reported that NAA (3.0 uM) resulted in 80% rooting but plantlets lacked vascular connections between roots and shoots which restricted acclimation. Driver and Kuniyuki (1984) dipped the basal end of 'Paradox' walnut shoots produced in vitro in IBA and placed them directly into a mixture of sand, peat and perilite (1:1:1). They were then placed under mist, at 85% relative humidity, where rooting occurred in 10 to 14 days. Driver and Kuniyuki (1984) reported that the optimum level of IBA was 5 uM.

The establishment of rooted shoots in soil requires a highly humid environment to prevent wilting and death of the plantlet. This environment may be achieved by covering the rooted plantlet with a glass or plastic tent for the first two weeks in soil. Dodd (1983) found that rooting in vitro

was faster and more prolific when a filter paper bridge was used with liquid media, compared to solid media for, Prunus and Pyrus species.

A number of research projects have succeeded in rooting micropropagated shoots using embryonic tissue from black walnut (Caruso, 1983, Heile-Sudholt et al., 1986 and Cornu, 1988A).

Heile-Sudholt et al. (1986) were the only investigators to report rooting of black walnut microshoots from tissues older than mature embryos. They reported initially rooting 10 microshoots with 3 surviving six months in the greenhouse. The basal end of microshoots were subjected to 15 second dips in 10 mM IBA dissolved in 50% (v/v) ethanol and deionized water. Microshoots were then placed in 240 ml sterile baby food jars containing 100 cc vermiculite and 70 ml deionized water. Humidity was maintained at 100% for one week. Lids were removed for the second week. After this time, rooted microshoots were transplanted to 2:1:1 (v/v/v) peat: vermiculite: perilite in 10 cm standard polyethylene pots.

Stefan and Millikan (1986) were unable to promote root growth on micropropagated shoots generated from mature black walnut explants. They examined both solid and liquid media, reduced media strengths (1/2 and 1/10) and a variety of soil mixtures (vermiculite, vermiculite: peat (1:1), and vermiculite:perilite (1:1)). Several pretreatments were

examined including the application of IBA in talc (0.1, 0.3 and 0.8%) to shoots. One second dips in IAA, IBA and NAA were also tried without success. Stefan and Millikan (1986) were unable to root micropropagated shoots and ultimately resorted to grafting shoots to germinated walnut seeds.

CHAPTER III

METHODS

Preliminary Trials

Preliminary trials were conducted to examine alternate culture methods (McGranahan et al., 1987, Heile-Sudholt et al., 1986, Stefan and Millikan, 1983, 1985, 1986 and Stefan, 1989) and establish effective micropropagation techniques for black walnut for this study.

The physiologic state of the donor plant was an important factor in preliminary trials. In the first trial, explants were isolated in October of 1987, using black walnut stump sprouts as the tissue source. This trial compared solid and liquid DKW-C media. Twenty nodal segments containing a single lateral bud were placed on each of the two media types. Severe (100%) contamination and no evidence of growth were apparent after two weeks and the trial was concluded. Explant tissues placed directly on solid DKW-C media had more rapid necrosis than those placed in liquid media. After two days in culture, an unsuccessful attempt was made to re-surface sterilize explants by 10 second dips in 20% clorox followed by two rinses in sterile water. Dips in 1% Daconil 2787 (Ortho) were attempted

without success. Gelrite was found to be difficult to dissolve in the media and no satisfactory solution was identified during this experiment. Seasonal timing was considered a primary factor in the observed high contamination levels and lack of growth. Explants were physiologically juvenile material but were isolated from dormant plants in the field.

A solution to lumping problems with Gelrite was identified based on the manufacturers recommendation. When Gelrite was mixed with sucrose and 0.2 g/l magnesium chloride ($MgCl*6H_2O$) and water was added gradually, lumping problems were minimized.

In the fall of 1988, further trials were conducted with local seedling material (two years old) to further assess culture methods. One year old seedling were removed from cold storage and planted in early June. Aseptic technique was evaluated during preliminary work. Twenty one nodal explants were isolated and surface sterilized in early September and place in liquid media for the first six days in culture. Explants were transferred to solid media after this time. After three weeks, 18/20 explants were contaminated and showed no signs of growth. In late September, 41 more nodal and terminal bud explants were isolated. Foam plugs were added to liquid media to prevent explant submersion. Contamination appeared to have initiated from dormant terminal and lateral buds. Explants

with terminal buds showed signs of growth. Within two weeks, 75% were contaminated. 100% explant death was observed within three weeks. Contamination (presumed to be primarily internal) was identified as the most extreme problem in the micropropagation of black walnut.

In March of 1989, further micropropagation trials were conducted with rapidly growing two year old black walnut seedlings. Twenty one nodal and terminal bud explants were place on liquid DKW-C media. The water used in the media and during isolation was changed from double distilled deionized to reverse osmosis water processed by a Nanopure system™ (Barnsted). Terminal bud expansion and lateral bud swell occurred by day 8 in culture. Within 14 days of explant initiation, 75% of explants were contaminated but the contaminants did not appear phytotoxic in all cases. Contamination was identified to be bacterial and nonpathogenic by the Plant Disease Diagnostic lab at Oklahoma State University, which recommended adding streptomycin to the media. Explant growth was limited to the expansion of single leaves.

In late March, 25 additional explants were isolated. Ten surviving explants started in early March and 21 started late March were placed on solid DKW-C media containing 125 mg/l streptomycin after 24 and 8 days in culture. Streptomycin appeared to restrict some contaminant growth. Leaf edge necrosis became evident within five days after

transfer to solid DKW-C containing streptomycin. After two weeks on DKW-C with streptomycin explants were transferred to media containing 125 mg/l streptomycin and 125 mg/l oxytetracycline. This medium did not solidify completely and was brown in color. Explants showed extensive necrosis within 5 days and were transferred to DKW-C with no antibiotics. Some contamination persisted while no further growth was evident. Explants did not recover and trials were concluded.

Based on these trials and review of the literature, a set of micropropagation techniques was selected. Black walnut seedling material was chosen for this study of in vitro variation to maximize morphogenic potential and limit internal contamination. Shoot tips of two month old black walnut seedlings were chosen as an explant source based on the success of previous work with walnut and for ease of isolation. The use of seedlings from a common environment reduced environmental variation and internal contamination, and provided rapidly growing and differentiating tissue.

Explants were isolated directly from the donor seedlings in the lab to eliminate contamination during storage. Two month old seedlings should have had less internal contamination than older tissues. A surface sterilization technique similar to that used by Heile-Sudholt et al. (1986) was chosen for this study based on their success with black walnut seedlings. Explants were

first soaked for 15 minutes in 0.50% NaOCl followed by three one minute rinses in sterile filtered water.

DKW-C medium was chosen for this study of in vitro variation based on the work of Stefan and Millikan (1984, 1985 and 1986) and Heile-Sudholt et al. (1986) using black walnut. A rapid transfer technique similar to that used by Heile-Sudholt et al. (1986) and employed successfully during preliminary trials was used to reduce the effects of phenolic production following explant isolation. Explants were placed in liquid media for the first six days and transferred to solid DKW-C after that time.

This study used DKW-C media which contained 4.4 uM (1.0 mg/l) BAP and .05 uM (.01 mg/l) IBA for shoot induction. No antibiotics were included in media, as no satisfactory combination of antibiotics has been identified. Gelrite was selected as a gelling agent for DKW-C media for this study because this media was designed to include gelrite. Gelrite does not adsorb compounds or contain the contaminants that agar does.

Experimental Design

In order to examine geographic variation in the micropropagability of black walnut in vitro, seed source collection sites were selected using a systematic sampling design. A grid was placed randomly over a map of black walnut's natural range and 16 single county collection sites

were identified. In September 1988, letters requesting seed collections were sent to potential cooperative researchers near the selected sites.

The seed collection requests asked for 50-100 seeds from five trees selected randomly in natural stands from each site. Selected trees were to be separated by a minimum of 100 meters to ensure the collection of a representative sample of local genotypes. Of the 16 seed collections requested, six were received and used in this study.

All seeds were stored at four degrees Celsius for 210 to 270 days before planting.

In July of 1989, 48 seeds from each of the 30 families collected were planted. Family numbers were assigned at this time based on the planting order. Seeds were planted individually in quart containers in a potting mix of approximately 10% peat, 10% vermiculite, 10% perilite and 70% top soil and grown in a greenhouse.

In Vitro Culture Methods

This study of in vitro variation in the micropropagability of black walnut examined seedlings from six locations, sampling five families of open pollinated seeds per location. The seed source collections included sites in Indiana, Iowa, Missouri, Oklahoma, Pennsylvania, and Tennessee (FIGURE 1). The black walnut families involved in this study were limited by seed collections

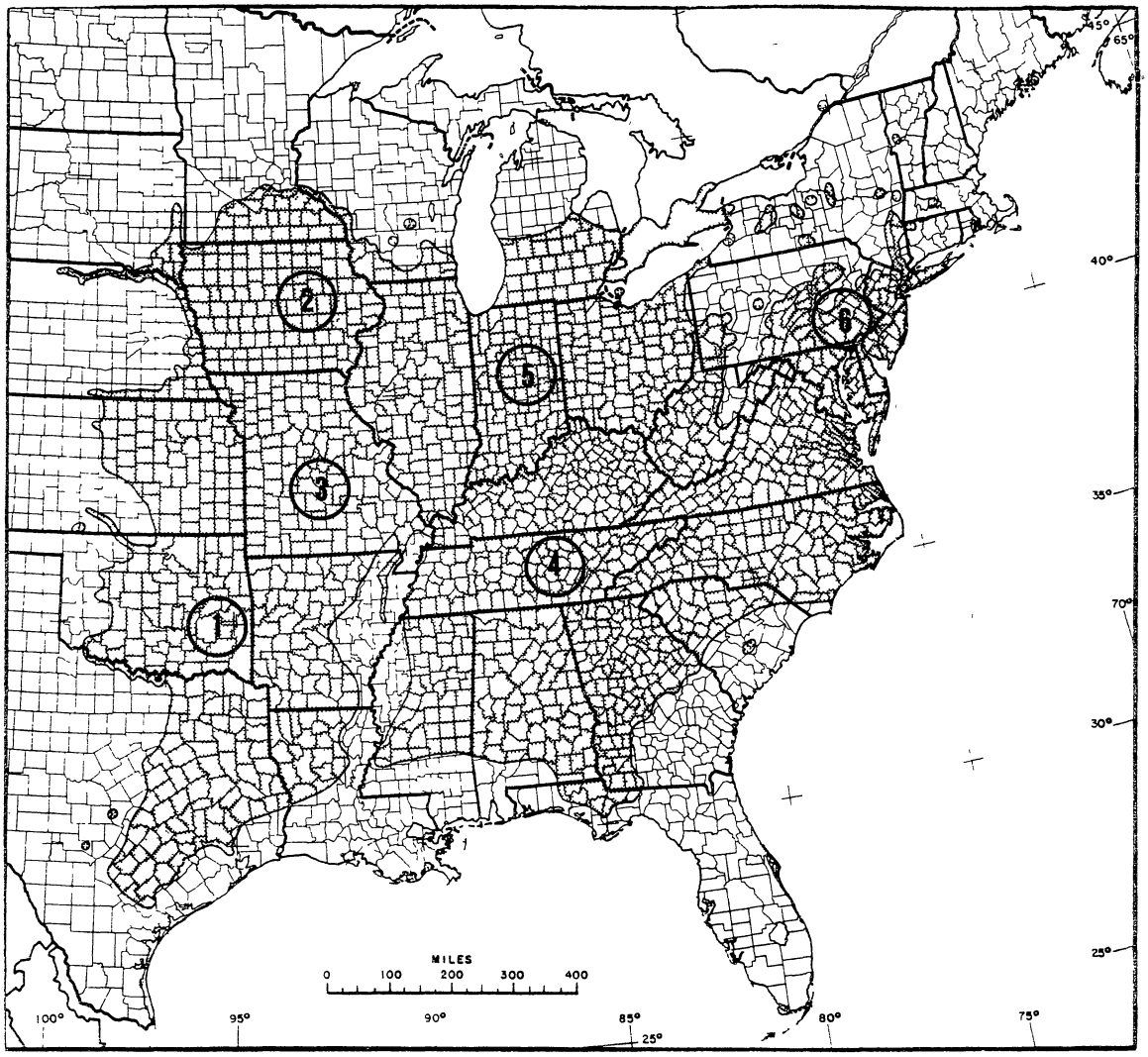

FIGURE 1. Black Walnut Range and Seed Collection Map

(Reprinted from Fowells (1965))

received and germination success.

The micropropagation experiment was divided into four replicates. Each replicate consisted of single terminal bud explants from each of five seedlings per family. The number of families included in each successive replicate decreased based on the number of seeds germinated.

Single terminal bud explants were isolated from vigorously growing seedlings (one explant/seedling) ranging in age from five to eight weeks. Shoot tips approximately 3-5 cm long containing a set terminal bud and 1-2 lateral buds were used for the explant tissue. All leaves were removed prior to surface sterilization. Seedlings with expanding terminal buds were avoided when possible. Initially, 10% of all explants had expanded terminal buds.

The surface sterilization procedure used was as follows:

1. a 15 minute rinse in 0.5% NaOCl containing 2 drops Tween 20 detergent
2. 3, 1-minute rinses in sterile nanopure water
3. recut the basal end of the explant.

Each family was surface sterilized separately to maintain family integrity.

Once an explant was surface sterilized, it was placed in a sterile environment. All materials used after this point were sterile. Transfers were made in a laminar flow hood using proven sterile technique. Culture tubes were

labeled with family and sample numbers, color coded by family, and autoclaved for 20 minutes at 121 ^{o}C. Explants were established in 10-12 ml of liquid basal DKW-C media (containing no plant growth regulators) (APPENDIX A) with 20 ml/l coconut water in culture tubes (25 X 150 mm). The basal liquid media was placed in culture tubes with foam plugs (1 X 3 cm) saturated with liquid media to maintain the vertical orientation of the explant and prevent explant submersion. Tubes were equipped with plastic caps with a foam plug insert to allow gas exchange but prevent contamination from entering.

Explants were maintained for the first 6 days on liquid basal media and were transferred to new media every two days over that period. Following the 6 day initiation period, explants were placed on solid DKW-C shoot induction media with 0.24% (2.4 g/l) Gelrite, 0.2 g/l $MgCl*6H_2O$, 4.4 uM (1 mg/l) BAP, .05 uM (.01mg/l) IBA and 2% (20 ml/l) coconut water. The plant growth regulators (IBA and BAP) and coconut water were filter sterilized and added to the rest of the medium after it was autoclaved for 20 minutes at 121 degrees Celsius in 900 ml volumes. The medium was then mixed thoroughly and 10-12 ml aloquots were placed into sterile culture tubes prior to media solidification.

Explants were transferred to new solid media on a biweekly basis following the initial six day period. Vertical orientation of the explant was maintained by basal

insertion in the media. When explants grew too large for the culture tubes, they were transferred to GA-7 Magenta boxes (Sigma) with 40-50 ml of solid shoot induction media.

Explants were grown in a growth chamber under 24 hr cool white fluorescent light for shoot growth. The temperature was maintained at 25 degrees Celsius for the shoot initiation period.

Scoring Methods

Each explant was scored every other day for growth and necrosis using an index, and for percent contamination over the first twenty days, followed by weekly scoring for the remainder of the project. After the first twenty days, changes in the growth scores were no longer occurring rapidly, prompting the less frequent scoring. Explants were scored for growth by visual examination using the following index (Cotton, 1983):

0 - Death of Explant

1 - Browning of Explant

2 - Yellowing of Explant

3 - Green Apex

4 - Swollen Apex

5 - Shoot Apex Expansion

Each individual was also scored visually for tissue necrosis using the following scale (Cotton, 1983);

0 - No Necrosis

1 - Tip of Meristem Necrotic

2 - 1/2 Meristem Necrotic

3 - All of Meristem Necrotic

4 - Most of Explant Necrotic

5 - Total Explant Necrosis

In addition, visible contamination of the explant and it's media were scored on a percentage basis. This score was not concisely definable, so presence or absence of contamination was used for statistical evaluation.

Rooting Procedures

The rooting procedures followed were similar to those described by McGranahan et al. (1987) for 'Paradox' walnut. Micropropagated shoots were placed on a rooting medium which contained adjusted levels (from DKW-C shoot induction media) of NH_4NO_3 (5.7 mM), $Ca(NO_3)*4H_2O$ (2.7 mM) and sucrose (153.8 uM) and the addition of 0.74 uM IBA. The light period was reduced from 24 to 16 hours and the night temperature was set at 18 degrees Celsius. After 7 days on rooting medium, shoots were washed in a slurry of systemic fungicide (Tersan 1991 DF (Dupont) and Bravo W75 (Diamond Shamrock Inc.), 1 tsp/l each) and the bases were dipped in a rooting powder containing 2 g IBA and 98 g talc (2% IBA). The shoots were then placed in peat and enclosed in a plastic bag to maintain 100% humidity for one week. Humidity was gradually

reduced by opening the seal over a period of five days. The humidity was maintained at 80% for four weeks following removal from plastic bags.

Statistical Methods

The growth, necrosis and contamination data collected during this study were analyzed using the Statistical Analysis System (Statistical Analysis System, 1985) computer program. An analysis of variance was conducted using the General Linear Model (GLM) function of SAS for each variable at each scoring period. An example of a general linear model for growth index on day 12 is given in TABLE I.

Location means were subjected to Least Significant Difference (t-test (LSD)) multiple comparison procedures for each variable at each time period. Comparisons were consider significant at the 0.05 level. In addition, location means for germination and lateral bud expansion were subjected to t-test (LSD) comparison.

The linear model examined variation due to location (LOC), family within location (FAM(LOC)), replicate (REP) and replicate by family within location (REP*FAM(LOC)). Due to unequal sample sizes and missing data, the Type III sum of squares model was used.

TABLE I

AN EXAMPLE OF AN ANALYSIS OF VARIANCE USING BLACK WALNUT EXPLANT DATA FROM DAY 12 FOR GROWTH INDEX USING THE GENERAL LINEAR MODEL PROCEDURE

Source	DF	SS	MS	F Value	PR>F	R-Square
Model	70	62.65	0.895	1.09	0.313	0.212
Error	283	232.80	0.823			
Corrected Total	353	295.45				
Loc	5	5.71		1.93	0.089	
Fam(Loc)	16	25.80		1.96	0.016[a]	
Rep	3	3.37		1.37	0.253	
Rep*Fam(Loc)	46	28.75		0.76	0.870	

[a] Significant at the 0.05 level

Model

Source	Type III Expected Mean Square
Loc	VAR(ERROR) + 4.99 VAR(REP*FAM(LOC)) + 14.43 VAR(FAM(LOC)) + Q(LOC)
Fam(Loc)	VAR(ERROR) + 4.99 VAR(REP*FAM(LOC)) + 15.77 VAR(FAM(LOC))
Rep	VAR(ERROR) + 4.98 VAR(REP*FAM(LOC)) + 81.36 VAR(REP)
Rep*Fam(Loc)	VAR(ERROR + 4.98 VAR(REP*FAM(LOC))

CHAPTER IV

RESULTS AND DISCUSSION

Statistical Analysis

The statistical analysis of the data, summarized in TABLE II, found significant location differences in the three scored explant characteristics. General linear models were constructed for each of the measured variables over the 18 scoring periods. Location, family and replicate were identified as significant sources of variation over the majority of this study (TABLE II). Only those sources of variation whose probability of a higher F value were below 0.05 were recorded in TABLE II and considered significant. The probability of a higher F value is the number entered in the table. It is interesting to note that no source of variation remained significant for the duration of the study. Once location became a significant source of variation for necrosis and growth it remained significant for the duration of the study. The fact that location, family, replicate and interaction term (Rep*Fam(Loc)) were all significant sources of variation in growth, necrosis and contamination *in vitro* support the findings of Heile-Sudholt et al. (1986) and Jay-Allemand et al. (1988B). These

TABLE II

BLACK WALNUT EXPLANT DATA SUMMARY:
PROBABILITY OF A HIGHER F VALUE
< 0.05 USING GENERAL
LINEAR MODELING

Day	Necrosis			Growth			Contamination		
	Loc	Fam	Int[1]	Loc	Fam	Int[1]	Loc	Fam	Int[1]
2			.0422	.0020		.0214			
4				.0001	.0074	.0152			
6				.0001	.0144				
8				.0001	.0091	.0216			
10				.0029			.0001	.0016	.0019
12	.0029	.0175			.0157		.0001	.0005	.0007
14	.0001	.0001		.0039	.0001		.0001	.0009	.0017
16	.0001	.0001		.0001	.0001		.0001	.0015	.0010
18	.0001	.0001		.0001	.0001		.0001	.0020	.0004
20	.0001	.0001		.0001	.0001		.0001	.0050	.0004
27	.0001	.0001	.0315	.0001	.0001	.0401	.0012	.0078	.0001
34	.0001	.0001	.0087	.0001	.0008		.0015	.0033	.0001
41	.0001	.0002		.0001	.0011	.0368		.0022	.0002
48	.0001	.0127		.0001				.0010	.0001
55	.0001			.0001		.0429			.0015
62	.0001			.0001					
69	.0001		.0020	.0064					
76	.0001		.0005	.0055		.0121			

[1] Rep*Fam(Loc)

studies reported finding intraspecific variation in growth and rooting in vitro for black walnut. The results of this study suggest that variation in growth and contamination do exist on a geographic basis.

Germination

The collected seeds were planted in quart containers July 6-12, 1989 and placed in a greenhouse. Seeds began to germinate in late July and continued to germinate through the middle of August. No germination occurred after August 31, 1989. Family and location germination data are summarized in TABLE III.

Variation in percent germination among locations was apparent. Location percent germination ranged from 5 to 47%. Seeds from Iowa (Location #2) had a significantly lower percent germination than the other five locations based on t-tests (LSD) with a 0.05 significance level.

A wide range of percent germination was observed (0-77%) among families. Seed of four out of the 30 open pollinated seed collections (half sib families) did not germinate. Variation in percent germination among families could have resulted from pre-stratification treatments (removal of the husk, relative humidity during cold storage, etc.), effect of the maternal parent, genetic make up or environmental factors affecting the maternal parent prior to, and during seed production among other possibilities.

TABLE III

FINAL BLACK WALNUT SEED COLLECTION GERMINATION PERCENTAGES ON A LOCATION AND FAMILY BASIS

Location	Family	Germination		
		Number	%[a]	% by Loc
#1 - Oklahoma	1	26	54	43
	2	27	56	
	3	4	8	
	4	30	63	
	5	15	35	
#2 - Iowa	1	0	0	5[b]
	2	2	4	
	3	10	21	
	4	0	0	
	5	0	0	
#3 - Missouri	1	16	33	43
	2	19	40	
	3	11	23	
	4	21	44	
	5	35	73	
#4 - Tennessee	1	2	4	29
	2	23	48	
	3	5	10	
	4	3	6	
	5	37	77	
#5 - Indiana	1	26	54	47
	2	0	0	
	3	28	58	
	4	35	73	
	5	23	48	
#6 - Pennsylvania	1	14	29	43
	2	8	17	
	3	17	35	
	4	36	75	
	5	29	60	

[a] 48 seeds from each family were planted
[b] Location 2 was significantly different from the other five locations at the 0.05 level

By September first, 22 families had at least 5 germinated seedlings, the required number for one replicate. The second 5 seedling replicate included 20 families. Only 17 families produced enough seedlings for the third replicate and 13 families germinated the 20 seedlings required for a fourth replicate (TABLE IV). Replicates were initiated at weekly intervals starting September 5th, 1989. Each of the first three replicates required two days for establishment *in vitro*. The fourth replicate required a single day for initiation.

Explant Growth

Terminal bud explants were isolated during the first four weeks of September, 1989. The number of explants included in this study by location and family are given in TABLE IV. Explants were monitored over a period of ten weeks and scored using the growth index. Over the first 3-4 weeks, explants grew rapidly and showed low contamination levels. After a month in culture, growth rates began to drop slowly (FIGURE 2).

In November (55 to 69 days in culture), all remaining explants were placed on rooting media for one week prior to rooting attempts. Explant death occurred over a period of 2-3 months after placement in peat, with no evidence of root growth. The rooting pretreatment appeared to be responsible for some of the rapid decline in the growth index and the

TABLE IV

BLACK WALNUT SEEDLING TISSUES INCLUDED IN THIS STUDY OF IN VITRO VARIATION BY LOCATION AND FAMILY

Location	Family	Seedling Number
#1 - Oklahoma	1	20
	2	20
	4	20
	5	15
#2 - Iowa	3	10
#3 - Missouri	1	15
	2	15
	3	10
	4	20
	5	20
#4 - Tennessee	2	20
	3	5
	5	20
#5 - Indiana	1	20
	3	20
	4	19
	5	20
#6 - Pennsylvania	1	10
	2	5
	3	10
	4	20
	5	20

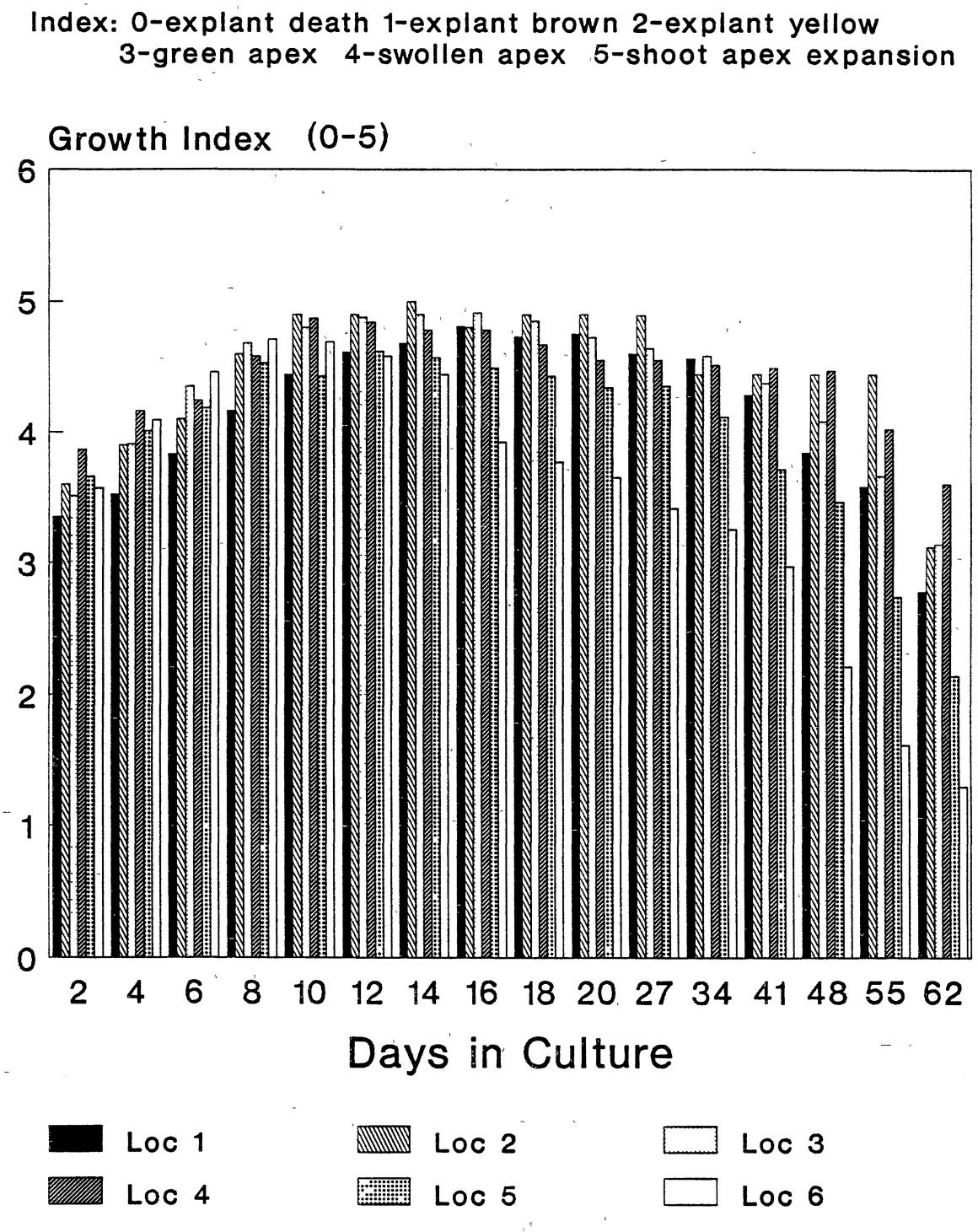

Figure 2. Explant Growth by Location Mean

increase in explant necrosis after day 55 (FIGURE 2).

Family and location differences were found to be significant for growth. The decline in the growth index appeared to be inversely related to contamination and necrosis levels. Other factors which may have affected the decline of the growth rate include the use of magnesium chloride (0.2 g/l, used to dissolve gelrite) and the lack of a chelated iron source in the media. Chelation of the iron source insures the continuous availability of iron to the explant. Dixon (1985) noted that iron depletion from the medium may occur rapidly in the absence of a chelating agent like ferrous-EDTA.

Contamination

The effect of contamination on the explant varied according to its rate of growth, preferred substrate (explant vs. media) and phytotoxicity. Specific contaminants were not identified in this study but could be visually separated into more than 10 types. Contamination growth rate and apparent substrate preference (explant vs. media) influenced the rate of explant necrosis. The rate of contaminant growth also influenced the timing of visual identification of contamination.

Visible contamination levels increased over the duration of the study from 0% on day 2 to 54% on day 55 (FIGURE 3 and APPENDIX B). Percent contamination by

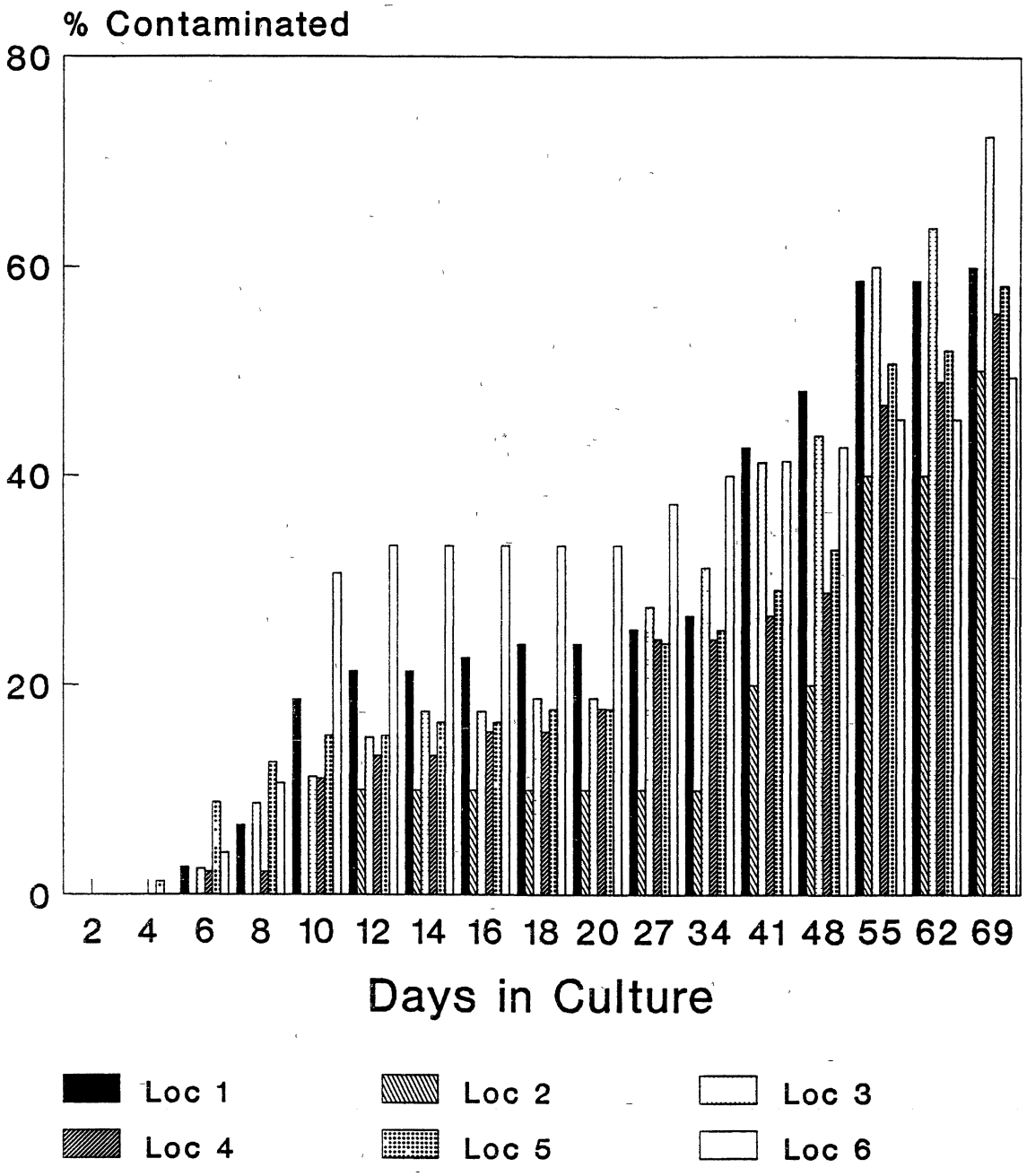

Figure 3. Explant Contamination by Location Mean

location was identified as significantly different at the 0.05 level using a t-test (LSD). Location 6 (Pennsylvania) was significantly different from locations 3, 4 and 5 from day 10 to day 18 (TABLE V). After this period, contamination levels at the other five locations increased to the level of location 6 and none were significantly different after day 34. Location differences may be, in part, the result of seed treatment, handling and cold storage which varied by supplier. Seeds from Missouri (#3) and Indiana (#5) were husked and float tested while the seeds from the other four location were placed directly in plastic bags and shipped.

Contamination appeared to arise from several parts of the explant. Initially, a few terminal buds may have harbored contaminants, particularly if they had begun to expand prior to explant isolation. In addition, the surface of the explant appeared to be the origin of contamination on a few other explants. This may indicate that surface sterilization techniques were insuffient or that contamination emerged through the bark or leaf scars of the explant. The final and most serious identifiable source of contaminant was internal, from the pith of the explant.

The effect of contamination on the explants in this study was dramatic. Contamination directly affected explant necrosis and growth and interfered with developmental comparisons. The lack of sustained rapid growth may have

TABLE V

BLACK WALNUT EXPLANT LOCATION MEANS IDENTIFIED AS SIGNIFICANTLY DIFFERENT BASED ON T-TESTS (LSD) OVER TIME

Means Significantly Different at the 0.05 Level			
Day	Necrosis	Growth	Contamination
2	1-4[a]		1-4,5
	3-4,5		3-4
4	3-5		1-3,4,5,6
6	3-4	3-5	1-3,4,5,6
8		4-5,6	1-3,4,5,6
10		6-3,4,5	1-3,4
			5-3,4
12	1-6	6-3,4,5	
14		6-3,4,5	
16	6-1,3	6-3,4,5	6-3
18	6-1,3	6-3,4,5	6-1,3
20	6-1,3	6-3,5	6-1,3
27	6-1,3,4,5		6-1,3,4
34	6-1,3,4,5	6-5	6-1,3,4
41	6-1,3,4,5		6-1,3,4
48	6-1,2,3,4,5		6-1,2,3,4
			4-5
55	6-1,2,3,4,5		6-1,2,3,4
	1-5		5-1,2,3,4
62	6-1,2,3,4,5		6-1,2,3,4
	1-5		5-1,3,4
			1-4
69	6-1,2,3,4		6-1,2,3,4
	5-1,2,3		5-2,4
			1-2
76	6-1,2,3,4		6-1,2,3,4
			5-2,4

[a] Necrosis index means for Locations 1 and 4 were identified as being significantly different using T-test (LSD) at the 0.05 level, this is how the remainder of this table should be interpreted.

prevented the identification of family and location differences in rooting and multiplication rate. As the contaminant grew, the explant's growth index generally declined as leaves and buds died. In addition, the explant's necrosis index increased over time as the explant began to die. Differences among families and locations in contamination rates may be a seed source limitation for commercial micropropagation purposes. Introduced contamination did not appear to be a significant problem in this study.

Necrosis

Explant necrosis was scored on a 0-5 scale with 0 being a healthy explant and 5 being complete explant necrosis. Location (TABLE V) and family (TABLE II) Index mean differences were identified using t-tests (LSD) and the application of the general linear modeling function of SAS.

Differences in the age of the donor seedling could affect susceptibility of tissue to the surface sterilization procedure. In addition, the extent of terminal bud expansion could affect the amount of tender tissue killed during the surface sterilization procedure. An effort was made to select seedlings with dormant terminal buds for explant isolation.

Initially, the necrosis index was close to zero (FIGURE 4) suggesting that expanded terminal buds were successfully

Index: 0-no necro. 1-meristem tip necro. 2-1/2 meristem necro. 3-meristem necro. 4-most of explant necro. 5-explant death

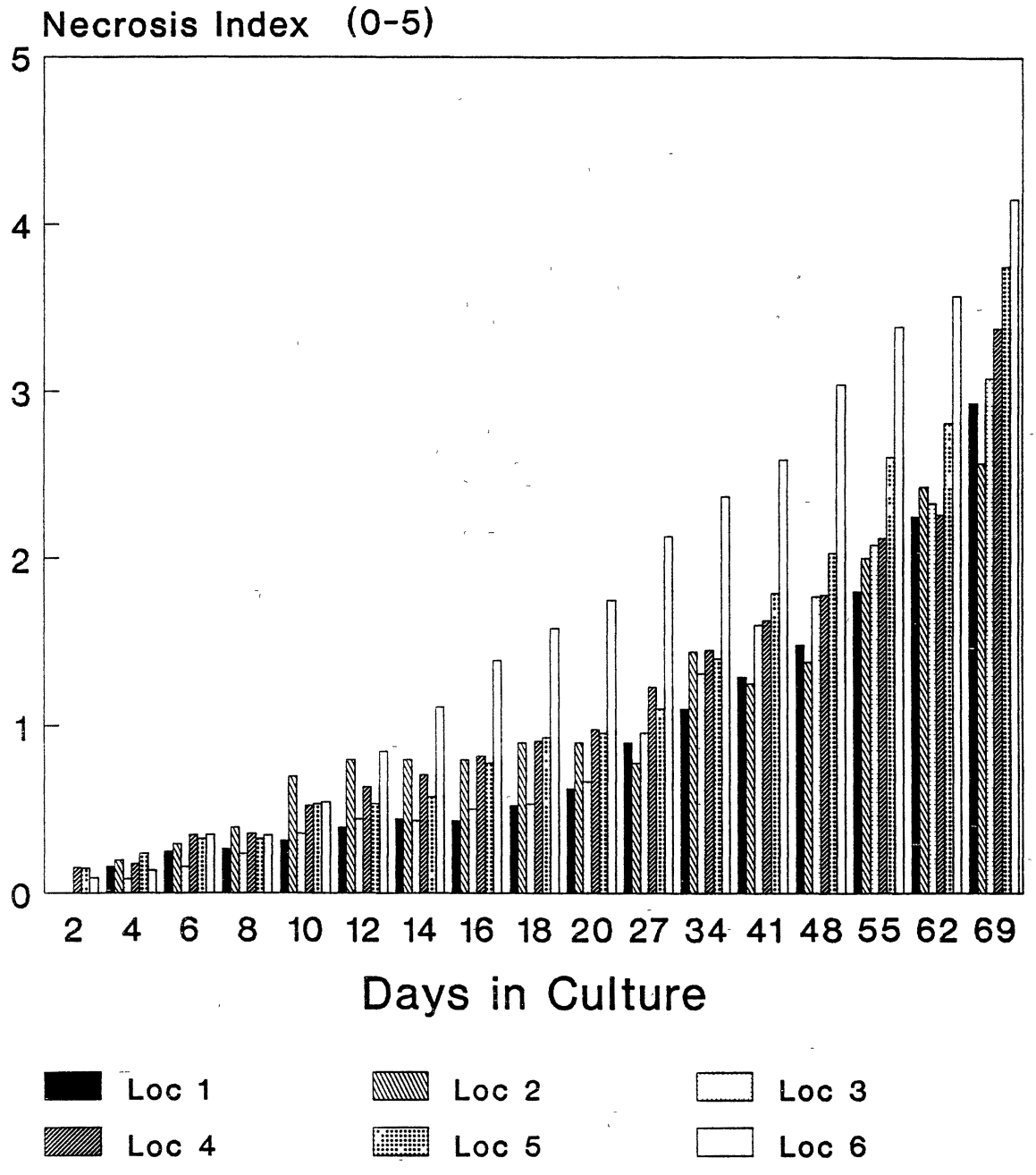

Figure 4. Explant Necrosis by Location Mean

avoided or that the surface sterilization procedure was not lethal to the explant. Necrosis gradually increased over the course of the study as contamination and other factors slowly killed some of the explants. When location means were subjected to a t-test (LSD), significance differences were observed and a trend appeared over the course of the study. The location 6 (Pennsylvania) index mean became significantly different from one other locations by day 12. By day 48, the necrosis index mean for location 6 was significantly different from all of the other five locations means. This trend corresponded fairly closely with the trend in percent contamination which gradually increased over the course of the study. As necrosis index and percent contamination increased at location 6 (Pennsylvania), growth index decreased.

Scoring Limitations

This study attempted to identify and document differences in growth and differentiation in vitro due to genetic differences among explant sources. Variation among geographic location of explant in growth rate and vigor were easily detected visually. However, the scoring system used did not adequately detect differences in relative vigor of the explant. For example, based on visual observation, all five families from Oklahoma (Location #1) appeared to grow more slowly and with less vigor than any of the other

locations when contamination was not a factor. Leaves were fewer in number, chlorotic and smaller, but the only difference in the index scores collected for these families was a slower increase in the growth index value over the first three weeks. An improved growth indicator might measure change in the fresh weight of the explant over time (Johnson, personal communication, 1990). The Oklahoma (location #1) means for the growth index were significantly different from three of the five other locations from day 10 to day 18 using t-test (LSD) (TABLE V). Significant differences in growth index means for location #1 were present from day 8 to day 34. While index scores were not significantly different after this time, growth continued to be slow and explants chlorotic. An improved scoring system should be more sensitive to indications of growth and vigor after the terminal bud had begun to expand.

One indication of relative vigor may be the expansion and growth of lateral buds. Families with slower growth rates or high contamination rates seem to have fewer expanded lateral buds (TABLE VI). Pennsylvania explants had high levels of phytotoxic contamination early in the culture cycle which lead to premature death. They usually did not live long enough to allow lateral bud expansion.

Plant Growth Regulators

The selection of plant growth regulators used for shoot

TABLE VI

LATERAL BUD EXPANSION ON *IN VITRO* EXPLANTS

Location	Family	Explants w/ Expanded Lat. buds (Total # of Explants Isolated)	% Expanded	% by Loc
#1 - Oklahoma	1	4(20)	20	23
	2	4(20)	20	
	4	5(20)	25	
	5	4(15)	27	
#2 - Iowa	3	7(10)	70	70
#3 - Missouri	1	6(15)	40	54
	2	9(15)	60	
	3	8(10)	80	
	4	7(20)	35	
	5	13(20)	65	
#4 - Tennessee	2	12(20)	60	56
	3	4(5)	80	
	5	9(20)	45	
#5 - Indiana	1	11(20)	55	47
	3	6(20)	30	
	4	11(19)	58	
	5	9(20)	45	
#6 - Pennsylvania	1	4(10)	40	28
	2	0(5)	0	
	3	0(10)	0	
	4	5(20)	25	
	5	9(20)	45	

initiation and rooting in this study was selected based on reports of successful micropropagation of 'Paradox' walnut (McGranahan et al., 1987). These plant growth regulator concentrations may be inappropriate or less than ideal for black walnut. The plant growth regulators used for shoot initiation were 1.0 mg/l BAP and 0.01 mg/l IBA. Stefan and Millikan (1985) used the following plant growth regulators for successful shoot initiation of black walnut: 4.0 mg/l BAP, 2.0 mg/l 2iP, 0.1 mg/l NAA and 0.000001 mg/l GA_3. They achieved more rapid shoot proliferation (10 fold in two months) than was evident in this study. The use of plant growth regulators optimized for 'Paradox' walnut may have been an error.

Rooting

Explants were placed on rooting media November 11th (Rep #1 {Day 66} and Rep #2 {Day 59}), November 14th (Rep #3 {Day 54}) and November 15th (Rep #4 {Day 48}). After one week on rooting media, explants were washed in a slurry of systemic fungicides (Tersan 1991 DF and Bravo W75, 1 tsp/l), dipped in 2% IBA and place in peat. Explants were maintained in a growth chamber with controlled day length, temperature and humidity. No root growth was observed before explant necrosis. Explants placed in peat for rooting purposes did not continue to produce leaves and tissue death occurred within several months. Lack of

sustained vigorous shoot growth prior to rooting attempts may have limited successful rooting. Heile-Sudholt et al. (1986) published the only report of rooted black walnut explants from tissue older than mature embryos. They produced rooted plantlet from seedling tissue. All other reports of successful production of rooted plantlets used embryonic tissue. Heile-Sudholt et al. (1986) initially rooted 10 shoots, but only 3 survived 6 months in a greenhouse.

Recommendations

A number of changes should be made in the culture media and the methods used in future work with black walnut micropropagation.

Contamination levels exceeding 50% within the first three months in culture are not acceptable. A number of changes in the culture cycle may lower the contamination rate or reduce its effects on the explant. The inclusion of a 2 minute soak in 70% ethanol in the surface sterilization procedure, similar to that used by Stefan and Millikan (1985), may improve results.

Isolation of meristems as opposed to shoot tips, should eliminate disease causing organisms which are transmitted through the vascular system (Meynier, 1985 and Stefan and Millikan, 1986). Flaming the mouth of the of the culture tube before and after any operation on the explant may

reduce introduced contamination. However, introduced contamination did not appear to contribute significantly to the overall contamination rate. Flaming is not an option with magenta boxes. The lid of magenta boxes should be sealed with felt tape to prevent entry of contamination while allowing gas transfer. Driver (personal communication, 1989) recommended transfers to new media on a weekly basis for better growth as opposed to the bimonthly transfers used in this study. Nutrient depletion may have been a factor in the lack of sustained rapid growth in vitro.

The inclusion of antibiotics in the media may reduce tissue necrosis due to contamination. Low levels of antibiotics (6-25 mg/l) similar to those used by Young (1984) should reduce or eliminate phytotoxic effects. Based on the preliminary work in this study, streptomycin was effective against a number of contaminants with no visible impact on the explant. Due to the large number of contaminant types encountered in this study, combinations of antibiotics may be necessary.

A number of changes in the media composition used may improve culture growth rates and reduce tissue necrosis not due to contamination. This study recommends the gelling agent, Phytagel (Sigma's version of gellum gum) as an alternative to gelrite. Phytagel should be added to the media after it is brought to volume and the pH is adjusted.

In addition, plant growth regulators should be changed to those used successfully with black walnut by Stefan and Millikan (1985). The placement of explants on a pretreatment media for rooting should be eliminated. It appeared to be detrimental to the explant.

CHAPTER V

SUMMARY AND CONCLUSIONS

Research in genetic variation *in vitro* and micropropagation methods of black walnut are important steps toward the improvement of black walnut for timber and nut production. Genetic variation in response to many factors of the micropropagation cycle may affect the application of this technique to black walnut tree improvement programs.

Although explants were not successfully rooted in this study, a number of significant goals were achieved. First, geographic variation in germination, explant growth, explant necrosis and contamination *in vitro* were identified. To the author's knowledge this is the first study that has examined geographic variation *in vitro* in *Juglans*. Secondly, differences in family and replicate were identified as significant sources of variation *in vitro*. Finally, internal sources of contamination in black walnut continued to emerge from the interior of the explant throughout this study. Internal contamination is a common problem in *Juglans* and work is needed to develop a method to isolate clean tissue.

Further work is needed to produce a successful rooting

procedure for both juvenile and mature tissue sources of black walnut. Only 10 micropropagated plantlets have ever been rooted *in vitro* using explant tissues older than mature embryos with black walnut (Heile-Sudholt et al., 1986). In addition, DKW media, designed specifically for 'Paradox' walnut, may need to be altered to provide optimal growth conditions for black walnut. Changes should include removal of magnesium chloride from the media and the use of the plant growth regulators and the levels that have been successful with black walnut in other studies.

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APPENDIXES

APPENDIX A

DKW-C MEDIA

Component	mM	mg/l
NH_4NO_3	17.7	1416.0
$Ca(NO_3)_2*H_2O$	8.3	1968.0
K_2SO_4	8.9	1559.0
$MgSO_4*7H_2O$	3.0	740.0
$CaCL_2*2H_2O$	1.0	149.0
KH_2PO_4	1.9	265.0
m-Inositol	0.55	100.0
Sucrose	87.6	30,000.0
	uM	
$Zn(NO_3)_2*6H_2O$	57.2	17.0
$MnSO_4*H_2O$	198.2	33.5
$CuSO_4*5H_2O$	1.0	0.25
H_3BO_3	77.6	4.8
$Na_2MoO_4*2H_2O$	1.6	0.39
$FeSO_4*7H_2O$	121.5	33.8
Na_2EDTA	135.0	45.4
$NiSO_4*6H_2O$	0.02	0.005
Thiamin*HCl	5.9	2.0
Nicotinic acid	8.1	1.0
Glycine	26.6	2.0

Media are adjusted to pH 5.4-5.7 and autoclaved for 20 minutes at 121 degrees Celcius.

(McGranahan et al., 1987)

APPENDIX B

BLACK WALNUT TISSUE CULTURE DATA BY LOCATION AND FAMILY

Day 2

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	0.10(20)	3.35(20)	0(20)
	2	0.05(20)	3.40(20)	0(20)
	4	0.10(20)	3.50(20)	0(20)
	5	0.07(15)	3.07(15)	0(15)
Location mean		0.08(75)	3.35(75)	0(75)
#2 Iowa	3	0.00(10)	3.60(10)	0(10)
#3 Missouri	1	0.07(15)	3.33(15)	0(15)
	2	0.00(15)	3.80(15)	0(15)
	3	0.00(10)	3.70(10)	0(10)
	4	0.00(20)	3.20(20)	0(20)
	5	0.15(20)	3.65(20)	0(20)
Location mean		0.05(80)	3.51(80)	0(80)
#4 Tennessee	2	0.25(20)	3.85(20)	0(20)
	3	0.00(5)	4.20(5)	0(5)
	5	0.10(20)	3.80(20)	0(20)
Location mean		0.16(45)	3.87(45)	0(45)
#5 Indiana	1	0.15(20)	3.75(20)	0(20)
	3	0.15(20)	3.65(20)	0(20)
	4	0.16(19)	3.47(19)	0(19)
	5	0.15(20)	3.75(20)	0(20)
Location mean		0.15(79)	3.66(79)	0(79)
#6 Pennsylvania	1	0.00(10)	3.80(10)	0(10)
	2	0.00(5)	3.80(5)	0(5)
	3	0.00(10)	3.50(10)	0(10)
	4	0.20(20)	3.40(20)	0(20)
	5	0.10(20)	3.60(20)	0(20)
Location mean		0.09(65)	3.57(65)	0(65)

[1] - number of explants scored
[2] - number of explants in study

Day 4

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	0.20(20)	3.50(20)	0(20)
	2	0.05(20)	3.55(20)	0(20)
	4	0.20(20)	3.80(20)	0(20)
	5	0.20(15)	3.13(15)	0(15)
Location Mean		0.16(75)	3.52(75)	0(75)
#2 Iowa	3	0.20(10)	3.90(10)	0(10)
#3 Missouri	1	0.07(15)	3.73(15)	0(15)
	2	0.00(15)	4.20(15)	0(15)
	3	0.10(10)	4.20(10)	0(10)
	4	0.05(20)	3.45(20)	0(20)
	5	0.20(20)	4.15(20)	0(20)
Location Mean		0.09(80)	3.91(80)	0(80)
#4 Tennessee	2	0.30(20)	4.20(20)	0(20)
	3	0.00(5)	4.60(5)	0(5)
	5	0.10(20)	4.10(20)	0(20)
Location Mean		0.18(45)	4.16(45)	0(45)
#5 Indiana		0.24(79)	4.01(79)	1(79)
	1	0.30(20)	4.00(20)	0(20)
	3	0.20(20)	4.00(20)	0(20)
	4	0.16(19)	3.95(19)	0(19)
	5	0.30(20)	4.10(20)	0(20)
#6 Pennsylvania	1	0.00(10)	4.00(10)	0(10)
	2	0.00(5)	4.40(5)	0(5)
	3	0.20(10)	3.90(10)	0(10)
	4	0.25(20)	4.15(20)	0(20)
	5	0.10(20)	4.10(20)	0(20)
Location Mean		0.14(65)	4.09(65)	0(65)

[1] - number of explants scored
[2] - number of explants in study

Day 6

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	0.30(20)	3.60(20)	5(20)
	2	0.10(20)	3.90(20)	0(20)
	4	0.30(20)	4.15(20)	5(20)
	5	0.33(15)	3.60(15)	0(15)
Location Mean		0.25(75)	3.83(75)	3(75)
#2 Iowa	3	0.30(10)	4.10(10)	0(10)
#3 Missouri	1	0.07(15)	4.27(15)	0(15)
	2	0.13(15)	4.73(15)	0(15)
	3	0.20(10)	4.50(10)	0(10)
	4	0.20(20)	4.05(20)	5(20)
	5	0.20(20)	4.35(20)	5(20)
Location Mean		0.16(80)	4.35(80)	2(80)
#4 Tennessee	2	0.40(20)	4.15(20)	5(20)
	3	0.20(5)	4.60(5)	0(5)
	5	0.35(20)	4.25(20)	0(20)
Location Mean		0.36(45)	4.24(45)	2(45)
#5 Indiana	1	0.40(20)	4.30(20)	15(20)
	3	0.30(20)	3.90(20)	15(20)
	4	0.16(19)	4.11(19)	0(19)
	5	0.30(20)	4.40(20)	5(20)
Location Mean		0.29(79)	4.17(79)	9(79)
#6 Pennsylvania	1	0.20(10)	4.70(10)	10(10)
	2	0.00(5)	4.60(5)	0(5)
	3	0.40(10)	4.60(10)	10(10)
	4	0.25(20)	4.25(20)	5(20)
	5	0.15(20)	4.45(20)	0(20)
Location Mean		0.22(65)	4.46(65)	5(65)

[1] - number of explants scored
[2] - number of explants in study

Day 8

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	0.30(20)	3.85(20)	5(20)
	2	0.15(20)	4.30(20)	0(20)
	4	0.30(20)	4.45(20)	10(20)
	5	0.33(15)	4.00(15)	13(15)
Location Mean		0.27(75)	4.16(75)	7(75)
#2 Iowa	3	0.40(10)	4.60(10)	0(10)
#3 Missouri	1	0.20(15)	4.67(15)	7(15)
	2	0.13(15)	5.00(15)	7(15)
	3	0.30(10)	4.70(10)	10(10)
	4	0.20(20)	4.40(20)	15(20)
	5	0.35(20)	4.70(20)	5(20)
Location Mean		0.24(80)	4.68(80)	9(80)
#4 Tennessee	2	0.25(20)	4.60(20)	5(20)
	3	0.20(5)	5.00(5)	0(5)
	5	0.50(20)	4.45(20)	0(20)
Location Mean		0.36(45)	4.58(45)	2(45)
#5 Indiana	1	0.35(20)	4.50(20)	15(20)
	3	0.40(20)	4.30(20)	15(20)
	4	0.26(19)	4.63(19)	11(19)
	5	0.30(20)	4.70(20)	10(20)
Location Mean		0.33(79)	4.53(79)	13(79)
#6 Pennsylvania	1	0.40(10)	4.80(10)	20(10)
	2	0.00(5)	4.80(5)	0(5)
	3	0.70(10)	4.80(10)	20(10)
	4	0.30(20)	4.50(20)	5(20)
	5	0.30(20)	4.80(20)	15(20)
Locatiom Mean		0.35(65)	4.71(65)	12(65)

1 - number of explants scored
2 - number of explants in study

Day 10

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	0.30(20)	4.10(20)	30(20)
	2	0.20(20)	4.55(20)	15(20)
	4	0.35(20)	4.55(20)	10(20)
	5	0.47(15)	4.60(15)	20(15)
Location Mean		0.32(75)	4.44(75)	19(75)
#2 Iowa	3	0.70(10)	4.90(10)	0(10)
#3 Missouri	1	0.40(15)	4.80(15)	20(15)
	2	0.27(15)	5.00(15)	7(15)
	3	0.70(10)	4.80(10)	10(10)
	4	0.25(20)	4.65(20)	15(20)
	5	0.35(20)	4.80(20)	5(20)
Location Mean		0.36(80)	4.80(80)	11(80)
#4 Tennessee	2	0.40(20)	4.85(20)	15(20)
	3	0.20(5)	5.00(5)	40(5)
	5	0.75(20)	4.85(20)	0(20)
Location Mean		0.53(45)	4.87(45)	11(45)
#5 Indiana	1	0.75(20)	4.20(20)	15(20)
	3	0.55(20)	4.20(20)	20(20)
	4	0.47(19)	4.84(19)	16(19)
	5	0.40(20)	4.50(20)	10(20)
Location Mean		0.54(79)	4.43(79)	15(79)
#6 Pennsylvania	1	0.70(10)	5.00(10)	40(10)
	2	0.80(5)	5.00(5)	100(5)
	3	0.90(10)	4.40(10)	50(10)
	4	0.45(20)	4.40(20)	15(20)
	5	0.35(20)	4.90(20)	30(20)
Location Mean		0.55(65)	4.69(65)	35(65)

[1] - number of explants scored
[2] - number of explants in study

Day 12

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	0.55(20)	4.25(20)	35(20)
	2	0.25(20)	4.85(20)	20(20)
	4	0.35(20)	4.60(20)	10(20)
	5	0.47(15)	4.80(15)	20(15)
Location Mean		0.40(75)	4.61(75)	21(75)
#2 Iowa	3	0.80(10)	4.90(10)	10(10)
#3 Missouri	1	0.47(15)	5.00(15)	20(15)
	2	0.27(15)	5.00(15)	13(15)
	3	0.80(10)	5.00(10)	10(10)
	4	0.35(20)	4.85(20)	20(20)
	5	0.50(20)	4.65(20)	10(20)
Location Mean		0.45(80)	4.88(80)	15(80)
#4 Tennessee	2	0.65(20)	4.70(20)	20(20)
	3	0.20(5)	5.00(5)	40(5)
	5	0.75(20)	4.95(20)	0(20)
Location Mean		0.64(45)	4.84(45)	13(45)
#5 Indiana	1	0.63(19)	4.35(20)	15(20)
	3	0.65(20)	4.55(20)	20(20)
	4	0.47(19)	5.00(19)	16(19)
	5	0.40(20)	4.60(20)	10(20)
Location Mean		0.54(78)	4.62(79)	15(79)
#6 Pennsylvania	1	1.30(10)	5.00(10)	40(10)
	2	2.20(5)	3.20(5)	100(5)
	3	1.20(10)	4.40(10)	70(10)
	4	0.55(20)	4.50(20)	15(20)
	5	0.40(20)	4.90(20)	35(20)
Location Mean		0.85(65)	4.58(65)	40(65)

[1] - number of explants scored
[2] - number of explants in study

Day 14

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	0.55(20)	4.30(20)	35(20)
	2	0.40(20)	4.90(20)	20(20)
	4	0.40(20)	4.70(20)	10(20)
	5	0.47(15)	4.87(15)	20(15)
Location Mean		0.45(75)	4.68(75)	21(75)
#2 Iowa	3	0.80(10)	5.00(10)	10(10)
#3 Missouri	1	0.40(15)	5.00(15)	20(15)
	2	0.40(15)	5.00(15)	13(15)
	3	0.80(10)	5.00(10)	10(10)
	4	0.40(20)	4.95(20)	20(20)
	5	0.37(19)	4.65(20)	20(20)
Location Mean		0.44(79)	4.90(80)	18(80)
#4 Tennessee	2	0.80(20)	4.75(20)	20(20)
	3	0.20(5)	5.00(5)	40(5)
	5	0.75(20)	4.75(20)	0(20)
Loacation Mean		0.71(45)	4.78(45)	16(45)
#5 Indiana	1	0.68(19)	4.40(20)	15(20)
	3	0.70(20)	4.35(20)	20(20)
	4	0.47(17)	5.00(17)	16(19)
	5	0.45(20)	4.60(20)	15(20)
Location Mean		0.58(76)	4.57(77)	16(79)
#6 Pennsylvania	1	1.70(10)	4.50(10)	40(10)
	2	4.20(5)	2.00(5)	100(5)
	3	1.40(10)	4.50(10)	70(10)
	4	0.60(20)	4.50(20)	15(20)
	5	0.37(19)	4.94(19)	35(20)
Location Mean		1.11(64)	4.43(64)	40(65)

[1] - number of explants scored
[2] - number of explants in study

Day 16

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	0.55(20)	4.55(20)	35(20)
	2	0.45(20)	4.95(20)	20(20)
	4	0.40(20)	4.85(20)	15(20)
	5	0.33(15)	4.93(15)	20(15)
Location Mean		0.44(75)	4.81(75)	23(75)
#2 Iowa	3	0.80(10)	4.80(10)	10(10)
#3 Missouri	1	0.47(15)	5.00(15)	20(15)
	2	0.40(15)	5.00(15)	13(15)
	3	0.80(10)	5.00(10)	10(10)
	4	0.55(20)	4.95(20)	20(20)
	5	0.42(19)	4.70(20)	20(20)
Location Mean		0.51(79)	4.91(80)	18(80)
#4 Tennessee	2	0.95(20)	4.75(20)	25(20)
	3	0.40(5)	5.00(5)	40(5)
	5	0.80(20)	4.75(20)	0(20)
Location Mean		0.82(45)	4.78(45)	16(45)
#5 Indiana	1	1.00(19)	4.25(20)	15(20)
	3	0.95(20)	4.15(20)	20(20)
	4	0.53(17)	5.00(17)	21(19)
	5	0.60(20)	4.65(20)	15(20)
Location Mean		0.78(76)	4.49(77)	18(79)
#6 Pennsylvania	1	2.10(10)	3.50(10)	40(10)
	2	5.00(5)	0.00(5)	100(5)
	3	1.90(10)	4.00(10)	70(10)
	4	0.70(20)	4.30(20)	15(20)
	5	0.53(19)	4.74(19)	35(20)
Location Mean		1.39(64)	3.92(64)	40(65)

[1] - number of explants scored
[2] - number of explants in study

Day 18

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	0.55(20)	4.55(20)	35(20)
	2	0.50(20)	4.95(20)	20(20)
	4	0.60(20)	4.55(20)	20(20)
	5	0.47(15)	4.93(15)	20(15)
Location Mean		0.53(75)	4.73(75)	24(75)
#2 Iowa	3	0.90(10)	4.90(10)	10(10)
#3 Missouri	1	0.47(15)	5.00(15)	20(15)
	2	0.40(15)	5.00(15)	13(15)
	3	0.90(10)	5.00(10)	10(10)
	4	0.60(20)	4.70(20)	20(20)
	5	0.47(19)	4.70(20)	20(20)
Location Mean		0.54(79)	4.85(80)	18(80)
#4 Tennessee	2	1.05(20)	4.50(20)	25(20)
	3	0.60(5)	5.00(5)	40(5)
	5	0.85(20)	4.75(20)	0(20)
Location Mean		0.91(45)	4.67(45)	16(45)
#5 Indiana	1	1.05(19)	4.25(20)	15(20)
	3	1.20(20)	3.90(20)	20(20)
	4	0.59(17)	5.00(17)	21(19)
	5	0.85(20)	4.65(20)	15(20)
Location Mean		0.93(76)	4.43(77)	18(79)
#6 Pennsylvania	1	2.40(10)	3.00(10)	40(10)
	2	5.00(5)	0.00(5)	100(5)
	3	2.00(10)	3.50(10)	70(10)
	4	0.85(20)	4.30(20)	15(20)
	5	0.79(19)	4.74(19)	35(20)
Location Mean		1.58(64)	3.77(64)	40(65)

[1] - number of explants scored
[2] - number of explants in study

Day 20

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	0.61(18)	4.56(18)	35(20)
	2	0.60(20)	4.95(20)	20(20)
	4	0.65(20)	4.55(20)	20(20)
	5	0.67(15)	5.00(15)	20(15)
Location Mean		0.63(73)	4.75(73)	24(75)
#2 Iowa	3	0.90(10)	4.90(10)	10(10)
#3 Missouri	1	0.53(15)	5.00(15)	20(15)
	2	0.47(15)	4.67(15)	13(15)
	3	1.30(10)	4.50(10)	10(10)
	4	0.75(20)	4.70(20)	20(20)
	5	0.53(19)	4.70(20)	20(20)
Location Mean		0.67(79)	4.72(80)	18(80)
#4 Tennessee	2	1.15(20)	4.25(20)	25(20)
	3	0.80(5)	5.00(5)	40(5)
	5	0.85(20)	4.75(20)	5(20)
Location Mean		0.98(45)	4.56(45)	18(45)
#5 Indiana	1	1.05(19)	4.25(20)	15(20)
	3	1.00(18)	3.85(20)	20(20)
	4	0.71(17)	5.00(17)	21(19)
	5	1.05(19)	4.37(19)	15(20)
Location Mean		0.96(73)	4.34(76)	18(79)
#6 Pennsylvania	1	2.50(10)	3.00(10)	40(10)
	2	5.00(5)	0.00(5)	100(5)
	3	2.67(9)	2.78(9)	70(10)
	4	0.90(20)	4.30(20)	15(20)
	5	0.95(19)	4.68(19)	35(20)
Location Mean		1.75(63)	3.65(63)	40(65)

[1] - number of explants scored
[2] - number of explants in study

Day 27

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	0.69(16)	4.38(16)	35(20)
	2	1.00(19)	4.95(19)	20(20)
	4	1.11(18)	4.33(18)	25(20)
	5	0.73(15)	4.73(15)	20(15)
Location Mean		0.90(68)	4.60(68)	25(75)
#2 Iowa	3	0.78(9)	4.89(9)	10(10)
#3 Missouri	1	0.92(13)	5.00(13)	27(15)
	2	1.00(12)	4.62(13)	13(15)
	3	1.40(10)	4.30(10)	10(10)
	4	0.89(18)	4.58(19)	35(20)
	5	0.76(17)	4.67(18)	30(20)
Location Mean		0.96(70)	4.64(73)	25(80)
#4 Tennessee	2	1.38(18)	4.21(19)	30(20)
	3	1.20(5)	5.00(5)	60(5)
	5	1.10(20)	4.75(20)	10(20)
Location Mean		1.23(43)	4.51(44)	24(45)
#5 Indiana	1	1.37(19)	4.25(20)	20(20)
	3	1.11(18)	3.90(20)	20(20)
	4	0.88(16)	5.00(16)	36(19)
	5	1.00(17)	4.37(19)	20(20)
Location Mean		1.10(70)	4.35(75)	24(79)
#6 Pennsylvania	1	2.80(10)	3.00(10)	40(10)
	2	5.00(5)	0.00(5)	100(5)
	3	3.00(8)	2.50(8)	70(10)
	4	1.25(20)	4.15(20)	20(20)
	5	1.56(18)	4.17(18)	45(20)
Location Mean		2.13(61)	3.41(61)	45(65)

[1] - number of explants scored
[2] - number of explants in study

Day 34

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	0.88(16)	4.31(16)	35(20)
	2	1.16(19)	4.84(19)	20(20)
	4	1.41(17)	4.33(18)	25(20)
	5	0.93(15)	4.73(15)	27(15)
Location Mean		1.10(67)	4.56(68)	27(75)
#2 Iowa	3	1.44(9)	4.44(9)	10(10)
#3 Missouri	1	1.00(13)	5.00(13)	27(15)
	2	1.50(12)	4.62(13)	13(15)
	3	1.80(10)	4.00(10)	10(10)
	4	1.39(18)	4.47(19)	40(20)
	5	1.06(17)	4.67(18)	45(20)
Location Mean		1.31(70)	4.58(73)	30(80)
#4 Tennessee	2	1.61(18)	4.21(19)	30(20)
	3	1.50(4)	5.00(4)	60(5)
	5	1.30(20)	4.70(20)	10(20)
Location Mean		1.45(42)	4.51(43)	24(45)
#5 Indiana	1	1.68(19)	3.65(20)	20(20)
	3	1.28(18)	3.90(20)	20(20)
	4	1.31(16)	4.94(16)	37(19)
	5	1.29(17)	4.11(19)	25(20)
Location Mean		1.40(70)	4.11(75)	25(79)
#6 Pennsylvania	1	2.80(10)	3.00(10)	40(10)
	2	5.00(5)	0.00(5)	100(5)
	3	3.12(8)	2.50(8)	70(10)
	4	1.58(19)	4.00(20)	20(20)
	5	1.80(15)	3.82(17)	55(20)
Location Mean		2.37(57)	3.25(60)	48(65)

[1] - number of explants scored
[2] - number of explants in study

Day 41

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	1.38(16)	3.94(16)	45(20)
	2	1.26(19)	4.26(19)	30(20)
	4	1.31(16)	4.06(17)	55(20)
	5	1.20(15)	4.93(15)	40(15)
Location Mean		1.29(66)	4.28(67)	43(75)
#2 Iowa	3	1.25(8)	4.44(9)	20(10)
#3 Missouri	1	1.46(13)	4.62(13)	33(15)
	2	1.58(12)	4.38(13)	27(15)
	3	1.90(10)	4.50(10)	30(10)
	4	1.76(17)	3.89(18)	50(20)
	5	1.31(13)	4.64(14)	50(20)
Location Mean		1.60(65)	4.37(68)	40(80)
#4 Tennessee	2	1.83(18)	4.21(19)	35(20)
	3	2.00(4)	5.00(4)	60(5)
	5	1.37(19)	4.65(20)	10(20)
Location Mean		1.63(41)	4.49(43)	27(45)
#5 Indiana	1	1.89(18)	3.32(19)	25(20)
	3	2.11(18)	3.25(20)	30(20)
	4	1.50(14)	4.86(14)	37(19)
	5	1.56(16)	3.74(19)	28(20)
Location Mean		1.79(66)	3.71(72)	29(79)
#6 Pennsylvania	1	3.00(10)	3.00(10)	40(10)
	2	5.00(5)	0.00(5)	100(5)
	3	3.38(8)	1.88(8)	80(10)
	4	1.95(19)	3.80(20)	15(20)
	5	1.75(12)	3.40(15)	60(20)
Location Mean		2.59(54)	2.96(58)	49(65)

[1] - number of explants scored
[2] - number of explants in study

Day 48

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	1.33(15)	3.79(14)	45(20)
	2	1.61(18)	3.63(19)	45(20)
	4	1.50(16)	3.82(17)	55(20)
	5	1.43(14)	4.21(14)	47(15)
Location Mean		1.48(63)	3.84(64)	48(75)
#2 Iowa	3	1.38(8)	4.44(9)	20(10)
#3 Missouri	1	1.54(13)	4.23(13)	33(15)
	2	1.92(12)	3.85(13)	27(15)
	3	2.00(9)	3.89(9)	30(10)
	4	1.93(15)	3.75(16)	60(20)
	5	1.54(13)	4.64(14)	50(20)
Location Mean		1.77(62)	4.08(65)	43(80)
#4 Tennessee	2	1.94(18)	4.21(19)	40(20)
	3	2.00(4)	5.00(4)	60(5)
	5	1.58(19)	4.60(20)	10(20)
Location Mean		1.78(41)	4.47(43)	29(45)
#5 Indiana	1	2.11(18)	3.47(19)	35(20)
	3	2.31(16)	3.32(19)	35(20)
	4	2.07(14)	3.64(14)	37(19)
	5	1.62(16)	3.47(19)	25(20)
Location Mean		2.03(64)	3.46(71)	33(79)
#6 Pennsylvania	1	3.50(10)	1.80(10)	40(10)
	2	5.00(5)	0.00(5)	100(5)
	3	3.71(7)	1.43(7)	80(10)
	4	2.42(19)	3.15(20)	15(20)
	5	2.36(11)	2.36(14)	60(20)
Location Mean		3.04(52)	2.21(56)	49(65)

[1] - number of explants scored
[2] - number of explants in study

Day 55

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	1.64(14)	3.57(14)	55(20)
	2	1.94(18)	3.53(19)	60(20)
	4	1.73(15)	3.65(17)	70(20)
	5	1.86(14)	3.57(14)	47(15)
Location Mean		1.80(61)	3.58(64)	59(75)
#2 Iowa	3	2.00(8)	4.44(9)	40(10)
#3 Missouri	1	1.67(12)	3.85(13)	40(15)
	2	2.08(12)	3.85(13)	60(15)
	3	2.44(9)	3.33(9)	60(10)
	4	2.40(15)	3.12(16)	65(20)
	5	1.84(13)	4.14(14)	65(20)
Location Mean		2.08(61)	3.66(65)	59(80)
#4 Tennessee	2	2.06(18)	4.21(19)	45(20)
	3	2.50(4)	2.50(4)	60(5)
	5	2.11(19)	4.15(20)	45(20)
Location Mean		2.12(41)	4.02(43)	47(45)
#5 Indiana	1	2.72(18)	2.79(19)	55(20)
	3	2.75(16)	2.47(19)	50(20)
	4	2.71(14)	3.21(14)	53(19)
	5	2.21(14)	2.63(19)	45(20)
Location Mean		2.61(62)	2.75(71)	51(79)
#6 Pennsylvania	1	4.00(10)	1.00(10)	40(10)
	2	5.00(5)	0.00(5)	100(5)
	3	4.17(6)	1.00(7)	80(10)
	4	2.72(18)	2.50(20)	25(20)
	5	2.70(10)	1.64(14)	65(20)
Location Mean		3.38(49)	1.61(56)	54(65)

[1] - number of explants scored
[2] - number of explants in study

Day 62

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	2.61(13)	2.50(14)	55(20)
	2	2.38(16)	2.79(19)	60(20)
	4	2.00(15)	3.24(17)	70(20)
	5	2.00(11)	2.50(14)	47(15)
Location Mean		2.25(55)	2.78(64)	59(75)
#2 Iowa	3	2.42(7)	3.12(8)	40(10)
#3 Missouri	1	2.09(11)	3.69(13)	53(15)
	2	2.18(11)	2.62(13)	60(15)
	3	2.62(8)	2.89(9)	70(10)
	4	2.50(14)	2.69(16)	90(20)
	5	2.31(13)	3.79(14)	65(20)
Location Mean		2.33(57)	3.14(65)	62(80)
#4 Tennessee	2	2.33(18)	3.84(19)	45(20)
	3	2.00(2)	2.50(4)	60(5)
	5	2.22(18)	3.60(20)	50(20)
Location Mean		2.26(38)	3.60(43)	49(45)
#5 Indiana	1	3.00(16)	2.15(19)	55(20)
	3	2.94(16)	2.16(19)	55(20)
	4	2.92(12)	2.31(13)	53(19)
	5	2.31(13)	2.00(19)	50(20)
Location Mean		2.81(57)	2.14(70)	53(79)
#6 Pennsylvania	1	4.00(8)	0.80(10)	40(10)
	2	5.00(5)	0.00(5)	100(5)
	3	4.60(5)	0.50(6)	80(10)
	4	3.00(17)	1.85(20)	25(20)
	5	2.89(9)	1.64(14)	65(20)
Location Mean		3.56(44)	1.29(55)	54(65)

[1] - number of explants scored
[2] - number of explants in study

Day 69

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	3.00(10)	1.29(14)	55(20)
	2	3.00(13)	1.47(19)	60(20)
	4	3.27(15)	1.47(17)	75(20)
	5	2.12(8)	2.21(14)	47(15)
Location Mean		2.93(46)	1.59(64)	60(75)
#2 Iowa	3	2.57(7)	2.87(8)	50(10)
#3 Missouri	1	3.00(11)	2.38(13)	67(15)
	2	3.11(9)	1.00(13)	67(15)
	3	3.14(7)	2.00(9)	80(10)
	4	3.31(13)	1.56(16)	70(20)
	5	2.85(13)	2.86(14)	75(20)
Location Mean		3.08(53)	1.95(65)	71(80)
#4 Tennessee	2	3.44(18)	2.16(19)	50(20)
	3	4.00(2)	1.25(4)	80(5)
	5	3.24(17)	2.10(20)	55(20)
Location Mean		3.38(37)	2.05(43)	56(45)
#5 Indiana	1	3.71(14)	1.47(19)	55(20)
	3	4.13(15)	1.21(19)	60(20)
	4	4.36(11)	1.00(13)	53(19)
	5	2.64(11)	1.84(19)	65(20)
Location Mean		3.75(51)	1.41(70)	58(48)
#6 Pennsylvania	1	4.50(8)	0.50(10)	60(10)
	2	5.00(5)	0.00(5)	100(5)
	3	4.60(5)	0.50(6)	90(10)
	4	3.62(13)	0.79(19)	25(20)
	5	3.78(8)	1.07(14)	65(20)
Location Mean		4.15(39)	0.70(54)	58(65)

[1] - number of explants scored
[2] - number of explants in study

Day 76

Location	Family	Index Means		Contam.
		Necrosis(#[1])	Growth(#[1])	%(#[2])
#1 Oklahoma	1	4.00(10)	1.07(14)	55(20)
	2	4.36(11)	0.68(19)	60(20)
	4	3.92(13)	1.00(17)	75(20)
	5	3.14(7)	1.43(14)	47(15)
Location Mean		3.92(41)	1.02(64)	60(75)
#2 Iowa	3	3.17(6)	2.25(8)	50(10)
#3 Missouri	1	4.09(11)	1.23(13)	67(15)
	2	4.17(6)	0.77(13)	67(15)
	3	4.00(7)	1.67(9)	80(10)
	4	4.38(13)	0.62(16)	70(20)
	5	3.38(13)	2.50(14)	75(20)
Location Mean		3.98(50)	1.32(65)	71(80)
#4 Tennessee	2	3.83(18)	1.89(19)	50(20)
	3	4.00(2)	1.25(4)	80(5)
	5	4.06(17)	1.40(20)	55(20)
Location Mean		3.95(37)	1.60(43)	56(45)
#5 Indiana	1	4.57(14)	0.68(19)	55(20)
	3	4.47(15)	0.68(19)	60(20)
	4	4.73(11)	0.38(13)	53(19)
	5	3.45(11)	1.58(19)	65(20)
Location Mean		4.33(51)	0.87(70)	58(79)
#6 Pennsylvania	1	5.00(7)	0.00(10)	60(10)
	2	5.00(5)	0.00(5)	100(5)
	3	5.00(5)	0.00(6)	80(10)
	4	4.08(13)	0.79(19)	25(20)
	5	5.00(8)	0.00(14)	65(20)
Location Mean		4.68(38)	0.28(54)	57(65)

[1] - number of explants scored
[2] - number of explants in study

VITA

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Master of Science

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