To the Graduate Council:

I am submitting herewith a thesis written by Denita Hadziabdic entitled "*In Vitro* Regeneration of *Cladrastis kentukea* (American yellowwood) and *Cornus kousa* (kousa dogwood). " I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

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In Vitro Regeneration of *Cladrastis kentukea* (American yellowwood) and *Cornus kousa* (kousa dogwood)

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee

Denita Hadziabdic
May 2005

DEDICATION

I would like to dedicate this work to my dad, the late Abdurahman Hadziabdic for his unconditional love, support, and never-ending faith in me. I wish you were here to share the joy of this incredible journey with me so I could, once again, make you proud. Thank you for beautiful memories.

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ABSTRACT

Selection of superior individuals followed by clonal vegetative propagation is a very important strategy for plant improvement. Cloning via tissue culture can produce a population of an identical genotype without limits. A single specimen tree of *Cladrastis kentukea* and five different *Cornus kousa* cultivars were selected for tissue culture studies. These trees exhibited superior performance in horticultural trials, including disease resistance and drought tolerance, which are highly important to the green industry.

Axillary buds from a single *C. kentukea* tree were initially cultured on either Woody Plant Medium (WPM) or Murashige and Skoog (MS) containing 0, 1, 2, or 4 µM 6–benzylaminopurine (BA). Cultures were transferred to fresh medium every four weeks. Elongated shoots were harvested after thirty-nine weeks and transferred to half-strength MS medium supplemented with following concentrations of IBA: 0, 3, 30, 100, and 300 µM for three days then returned to half-strength MS without growth regulators. Explants exposed to 300 µM of IBA produced significantly more roots (75%) compared to explants exposed to other treatments. Fifty- four and forty- six percent of the microshoots rooted when exposed to 100 and 30 µM IBA, respectively. Only 4% of the microshoots rooted when exposed to 3 µM IBA and none of the microshoots in the control treatment (0 IBA) rooted. Although 300 µM treatment yielded the most rooted plantlets, there was significantly higher terminal meristem abortion compared to other treatments. There were no statistical differences between the numbers of roots

and total root length among all treatments. Additionally, all microshoots that rooted had lenticels, suggesting that presence of lenticel cambial activity can possibly predict rooting abilities of selected microshoots. Rooted microshoots were gradually acclimatized to non-sterile environment.

Axillary and apical buds from five *Cornus kousa* cultivars (‘Little Beauty’, ‘Samaritan’, ‘Heart Throb’, ‘Rosabella’ and ‘Christian Prince’) were initially established on either WPM or one-half Woody Plant Medium/Broad Leaved Tree Medium (BW), amended with the following concentrations of 6–benzylaminopurine (BA): 0, 2, 4, and 8 µM. After explants were transferred at four-week intervals for 28 weeks beginning in April, only microshoots of ‘Samaritan’, ‘Heart Throb’, and ‘Rosabella’, were harvested from proliferating cultures and placed on rooting media. ‘Little Beauty’ and ‘Christian Prince’ did not perform well in multiplication phase of tissue culture and were excluded from further studies. Rooting media contained WPM or BW supplemented with either 1- naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), or indole-3-acetic acid (IAA) at the following concentrations: 0, 0.5, 1.5, 4.5, and 13.5 µM. Six weeks following rooting experiment, preliminary data was collected and results indicated a total of nine plants rooted on both WPM and BW media supplemented with IBA, 17 plants rooted on media supplemented with NAA, and 14 plants rooted plants supplemented with IAA. NAA and IAA appeared to be better for root production on *C. kousa* cultivars microshoots than IBA. Moreover, both WPM and BW media supported rooting of *C. kousa* microshoots. However, WPM appears to support more root production compared to BW. A greater

number of ’Samaritan’ and ‘Heart Throb’ microshoots rooted on WPM amended with a wide range of NAA concentrations, whereas more ‘Rosabella’ microshoots rooted on BW medium amended with various concentrations of IAA. Since ‘Rosabella’ and ‘Heart Throb’ are very closely related and should have rooted with similar treatments, further research is needed to confirm this finding. Additionally, microshoots placed on either basal media supplemented with NAA produced significant amount of callus compared to microshoots exposed to other growth regulator treatments. The highest mean number of roots per rooted microshoot was recorded on ‘Samaritan’ when exposed to various NAA concentrations. In conclusion, the most and best rooting occurred with IBA treatments at lower concentrations, 0.5 and 1.5 µM, whereas NAA and IAA treatments were inconclusive.

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Chapter One

Introduction

1.1 Plant Tissue Culture

1.11 Introduction to Plant Tissue Culture

Plant tissue culture and micropropagation are important alternatives to more conventional methods of plant propagation and involves producing, maintaining and establishing different plant organs (embryos, shoots, roots and flowers) and tissues (cells, callus and protoplasts) in axenic culture (Hartmann et al., 2002). Plant tissue culture offers a potential means of rapid mass multiplication of disease-free plants without seasonal constrains. However, an efficient, practical, and reliable method of propagation should be developed for the desired plant before commercial propagation by a tissue culture is economically and biologically feasible.

Although tissue culture techniques for many different species have been investigated, many protocols remain incomplete or deficient in producing viable, whole plants. Currently, the production of adventitious roots on shoots and whole plant acclimatization continues to be one of the primary obstacles that limits the overall success in many micropropagation systems (Newell et al., 2003).

Micropropagation uses a high degree of control over each aspect of regeneration and is employed as an accelerated form of clonal propagation, where resultant plants are genetically identical to parent plants. Murashige (1974) originally described only the following three basic stages for successful micropropagation: 1) establishment of aseptic cultures, 2) proliferation of axillary shoots, and 3) pretransplant (rooting). Today, because of better knowledge about plant morphology, physiology and genetic information, there are five stages of micropropagation. These are discussed in the following paragraphs.

Stage 0 - Donor plant selection and preparation

Explant quality and subsequent responsiveness in vitro are significantly influenced by the phytosanitary and physiological conditions of the donor plant (Debergh and Maene, 1981). Stock plants are maintained in clean and controlled environment by using best management and pest practices. This is however, done only in a timely manner when the benefits prevail over the costs and when taken actions are the most effective and least disruptive for the entire process. This procedure includes, but is not limited to, cultural, nutrient, pest, insect, disease, as well as biological management practices. Reducing pathogen activity and avoiding contamination of candidate plants generally will allow excision of relatively larger, healthier and more responsive explants. Additionally, many practices are employed to increase explant responsiveness by modifying the physiological status of the stock plan. These practices include the following: trimming to stimulate lateral shoot growth, pretreatment sprays containing cytokinins or gibberellic acid, as well as use of forcing solutions

containing 2% sucrose and 200mg/L 8-hydroxyquinoline citrate for induction of bud break and delivery of growth regulators to target explant tissues (Kane, 2000).

Stage I - Establishment of aseptic cultures

Initiation and aseptic establishment of pathogen free explants is the major goal of this stage of micropropagation. It is essential for stage I cultures to be indexed (screened) for the presence of internal microbial contaminants prior to serving as sources of shoot tip and nodal explants for stage II multiplication (Kane, 2000). There are many factors influencing and affecting the success of *in vitro* establishment of plant explants and they may include time, location on the plant and size of explant, maturity of the donor plant, and polyphenol oxidation. In most cases, explants are surface-sterilized prior to placement in culture.

There is no universal medium for establishment of plant explants for all species, but the most widely used basal medium is probably MS (Murashige and Skoog, 1962). In this stage, cytokinins and auxins are frequently added to medium to enhance explant survival and shoot development (Hu and Wang, 1983), and their concentrations are dependent on the species, genotype, and explant size (Kane, 2000). Many commercial laboratories maintain mother stock plants that are verified as being pathogen free of cultivable contaminants on media that limits shoot production to maintain genetic stability. These cultures serve as sources of shoot tips or nodal segments for initiation of new stage II cultures (Kane, 2000).

Stage II - Proliferation of axillary shoots

Stage II propagation is characterized by repeated enhanced formation of axillary shoots from shoot tips or lateral buds cultured on a medium supplemented with a relatively higher cytokinin (Kane, 2000). Shoot initiation is strongly supported by cytokinin concentration, however increased cytokinin levels may promote additional shoot proliferation, but can also inhibit shoot elongation. Auxin is usually maintained in low concentration or even absent in stage II cultures, but there are cases where auxin alone is used to induce shoot multiplication (Hartmann et al., 2002). Four to eight week subculturing intervals are commonly used in multiplication practices for many crops propagated by shoot culture. Nearly a month interval is used to maximize microshoots yield and enhance shoot proliferation of starting explants.

Stage III - Pretransplant (rooting)

Pretransplant rooting is focused on preparation of stage II shoots or shoot clusters for successful transfer into soil. This rooting process may involve elongation of shoots prior to rooting, rooting of individual shoots or shoot clumps, cold treatment of storage organs for dormancy purposed as well as prehardening cultures for increased survival (Kane, 2000). Many woody species require dipping of shoots in an auxin solution or auxin enriched medium to initiate rooting. The concentration and type of auxin used to induce root formation vary greatly between species. Extensive empirical experimentation may be required to select the most advantageous conditions for rooting.

Stage IV - Transfer to natural environment

Acclimatization of plantlets is the final stage of that determines the success of the micropropagation method. This process involves hardening of plantlets to conditions of significantly lower relative humidity and higher light intensity. Plants cultured *in vitro* have low photosynthetic activity as they have been cultured in the presence of sucrose and under low light and gas exchange conditions (Kane, 2000). They also have a poor control of water loss due to lack of leaf epicuticular wax, abnormal stomata functioning, poor vascular connections between roots and shoots, and poorly differentiated leaf mesophyll. Poor survival rates are frequently encountered even when all the necessary measurements and precautions are taken into account. In order to overcome these obstacles, rooted microshoots are transferred into a sterile (autoclaved) general-purpose medium and maintained under mist system and reduced light intensity. During this stage, plantlets are converted from a heterotrophic to photoautotrophic state (Preece and Sutter, 1991). Finally, transplants are gradually acclimatized to a non-sterile environment by increasing light intensity and low humidity conditions.

The objective of this project was focused on improving rooting efficiency and developing tissue culture systems for clonal propagation of two different ornamental species: *Cladrastis kentukea* (Dum.–Cours.) Rudd (Yellowwood) and *Cornus kousa* Hance (Kousa dogwood). This study provided new protocols for *in vitro* regeneration, rapid, more efficient and suitable propagation methods and a new approach to mass production of these valuable ornamental species.

Chapter Two

Cladrastis kentukea

2.1 Literature Review for *Cladrastis kentukea*

2.11 Cladrastis kentukea (Dum.–Cours.) Rudd Introduction

Cladrastis kentukea, the American yellowwood, is a beautiful, but little known American tree. It is rare in nature and cultivation, and is worthy in every respect of being considered one of the most beautiful trees of eastern North America (Robertson, 1977; Sand, 1992). *Cladrastis kentukea*, also known as *C. lutea* (Michx.f) K. Koch., is a member of the Fabaceae, subfamily Papilionoideae. This subfamily of the Fabaceae with pea–shaped flowers is easy to recognize as fossil flowers first appeared in the late Paleocene, together with more primitive mimosa-like flowers (Phillips and Rix, 2002). The *Cladrastis* genus encompasses only four species, of which three are native to southeastern Asia (Harrar, 1971), the remaining species is only found in North America.

The Fabaceae (formerly Leguminosae) is the third largest family of angiosperms, with three subfamilies comprising 650 genera and more than 18,000 species (Polhill and Raven, 1981). This family was erected by Bentham (1865) who separated member species of the Leguminosae according to their flower structure into the following three groups: Papilionoideae, Caesalpinioideae and Mimosoideae. Species classified in the Papilionoideae are characterized by

zygomorphic (bilaterally symmetrical) flowers that have the adaxial petal outside the adjacent lateral petals. The Caesalpinioideae species also have more or less zygomorphic flowers, but the adaxial petals are overlapped by the adjacent lateral petals. Finally, members included in the Mimosoideae are distinct from the first two groups because of their actinomorphic (radially symmetrical) flowers, which commonly have increased numbers of stamen (Kass and Wink, 1996). De Candolle (1825-27) proposed a closer relationship between two of the three subfamilies because of their overall similarities: Caesalpinoideae and Mimosoideae combined in one subfamily and Papilionoideae in another. According to Kass and Wink (1996), Papilionoideae and Mimosoideae are monophyletic whereas, Caesalpinioideae is paraphyletic to the other subfamilies. Therefore, the classical division of Leguminosae into three subfamilies was not supported by these authors.

Cladrastis kentukea is commonly referred to as American yellowwood or simply yellowwood because of its bright yellow heartwood and is the only North American species in the genus. It was named by the Frenchman, Andre Michaux (1746-1802), who was commissioned by the French government and Louis XVI to discover and introduce American trees to France for possible use by the royal navy (Sand, 1992). His son, Francois Andre, gathered yellowwood seeds and imported them to Tuileries, France, where apparently, descendants of those seedlings exist today (Robertson, 1977; Sand, 1992). Initially, yellowwood was referred as a new species of *Sophora* because of its unusual, smooth, silvery-

gray bark (Sand, 1992). In Francois' journal, written six years after yellowwood was discovered in 1796, he stated:

> "We found particularly, in these forests, a tree which, by the shape of its fruit and the disposition of its leaves, appears to have great affinity with the *Sophora japonica,* the wood of which is used by the Chinese for dyeing yellow. My father who discovered this tree in 1796, thought that it might be employed for the same use, and become an important object of traffic for the country. This tree very seldom rises above forty feet, and grows, in preference, on the knobs, species of little hills, where the soil is very rich. (Michaux, 1805)(Robertson, 1977; Sand, 1992)."

Francois Andre in his 1810-1813 three volume work, *Histoire des Arbres forestiers de l'Amerique septentrionale*, named yellowwood *Virgilia lutea* (Sand, 1992). Today, *Virgilia* is restricted to one or two species found in South Africa (Robertson, 1977). In 1824, C.S. Rafinesque published a new generic name *Cladrastis,* changing specific epithet to *tinctoria* (Robertson, 1977; Sand, 1992). Forty-five years later, German taxonomist and dendrologist, Karl H. Koch, had made another change by renaming *tinctoria* to *lutea*, still retaining Michaux's original epithet (Sand, 1992). Interestingly, two years prior to Michaux's official publication of *Virgilia lutea* in 1813, George Marie Louise Dumont de Courset had published a description of a young, nonflowering cultivated tree in France, naming it *Sophora kentukea* (Sand, 1992). Although the description appears to match the yellowwood, there are a number of differences among these species (Robertson, 1977). Finally, in 1971, V.E. Rudd, a botanist at the Smithsonian Institution renamed yellowwood to *Cladrastis kentukea*, based on Dumont de Courset description (Robertson, 1977; Sand, 1992).

Yellowwood is a moderately sized deciduous tree and can reach 10-15m in height with a spread of 12-15m. It has a habit of low branching with a broad, rounded crown of delicate branches (Dirr, 1998; Robertson, 1977). Planted at 2-2.5m, it becomes 5-6m tall with a nearly equal spread in 8-10 years (Crockett, 1972). The biggest tree in the United States was found in Cincinnati, OH and that national champion was 22 by 22m (Dirr, 1998).

Yellowwood is superb tree for many purposes. Its dense branches provide shade for patios, its deep roots do not disturb paving when the tree is planted along a street, and its fragrant white flowers add beauty anywhere (Crockett, 1972) (Figure 2.1.). One of the most striking and appealing attributes of this tree is its fall color change from dark green to glowing and breathtaking yellow. Species are sometimes planted as ornamentals and form rather open, upright trees (Phillips and Rix, 2002). The light gray bark is smooth, attractive and appealing, adding prominent value in a landscape especially when compared to the dark green of maples, oaks or ashes (Dirr, 1998).

2.12 Cladrastis kentukea Morphology

Yellowwood leaves are arranged alternately along the branches, odd-pinnately compound, approximately 20-30cm long with 5-11 leaflets (Dirr, 1998; Robertson, 1997) (Figure 2.1. and Figure 2.2.). The lateral leaflets are broadly elliptic or ovate in outline, with acuminate tips, slightly rounded bases with entire margins (Robertson, 1977). The terminal leaflet is more or less rounded to

Figure 2.1. Illustration of leaves, fruit and inflorescence of *Cladrastis kentukea*. Adapted from Britton, N.L., and A. Brown. 1913. Illustrated flora of the northern states and Canada. Vol. 2: 343. (USDA-NRCS, 2005)

Figure 2.2. *Cladrastis kentukea* thin, grayish to light brown bark color, one of the many attractive features of this tree (left) and alternately arranged, odd-pinnately compound leaves with inflorescences and fruits resembling flattened pod (right). Image adapted from J.S. Peterson @ USDA-NRCS PLANTS Database (USDA-NRCS, 2005)

rhomboid shape, with same acuminate tip, but has a cuneate base (Robertson, 1977). Opening leaf color is bright yellowish-green, which gradually changes to bright green in summer (Dirr, 1998) and in fall, leaves turn shades of yellow and orange before dropping (Crockett, 1972).

Yellowwood stems are slender, smooth, and odorous with bright reddish coloration (Dirr, 1998). It's bark is thin, gray to light brown color that resembles the bark of the beech tree, and remains beautiful even when the tree reaches maturity (Dirr, 1998) (Figure 2.2.). The genus *Cladrastis* is derived from Greek "klados" that means branch and "thraustos" meaning fragile (Robertson, 1977; Sand, 1992), describing the most prominent features of this tree. The braches are actually no more fragile than other trees, however, if pruned in seasons than summer, the tree can bleeds profusely (Crockett, 1972; Dirr, 1998).

Flowers are perfect, white, very fragrant, and usually pollinated by bees (Phillips and Rix, 2002). They are born 20-35cm long, 10-15cm wide at the base pendulous, *Wisteria*-like terminal panicles (Figure 2.2.) in May to early June (Dirr, 1998; Robertson, 1997). However, in Tennessee, yellowwood was found to start blooming in late April and early May (Trigiano, personal comm.). The flowers, 2.5cm long, are borne in clusters that hang from the ends of twigs (Crockett, 1972) and are quite fragrant, particularly in the evening (Robertson, 1977).

The sepals are united into a green tube with five calyx teeth and five petals are of three different sort – the upper one that encloses the two, lateral 'wing' petals and the two inner, 'keel' petals (Robertson, 1977). The outer petal

has a yellow area toward the base, attracting insect pollinators, whereas all other, white wing and keel petals serve as a base for insect landing (Robertson, 1977).

Yellowwood begins to blossom when 4.5-5.5m tall (Crockett, 1972), however, this tree is very slow growing, often taking ten to twenty years to flower for the first time. Typically, it flowers heavily only every second or third year (Robertson, 1977).

Yellowwood fruit is a flattened brown pea-like pod (Dirr, 1998) that mature in size by August or September and are brown and dry (Robertson, 1977). The fruit contains four to six (Dirr, 1998), olive brown and beanlike, hard coated seeds, slightly laterally flattened, approximately 0.6cm long and 0.4cm wide. It also resembles the fruit of redbud (*Cercis canadensis L.*) (Robertson, 1977).

The hardwood of this tree was used for gunstocks (Phillips and Rix, 2002). Lutea, one of the epithets given to this tree, is latin for yellow, referring to the yellow heartwood of the tree (Robertson, 1977; Sand, 1992). Both heartwood and the bark were used by the early American settlers to make yellow dye for homespun cloth (Crockett, 1972; Sand, 1992). Fortunately, for the sake of preserving this tree, the trunks of wild yellowwood trees fork too closely to the ground (Figure 2.3.) for the species to be used for commercial lumber industry (Robertson, 1977).

Figure 2.3. Thirty-five to forty year old *Cladrastis kentukea* tree in winter located on University of Tennessee campus in Knoxville, TN. Note the rounded crown and short trunk forked closely to the ground.

2.13 Cladrastis kentukea Culture

Yellowwood grows the best in full sun. The tree can tolerate almost any soil, including alkaline, as long as it is moist and deep enough to accommodate It's long root structure (Crockett, 1972). The species will even tolerate droughts, if the soil is watered thoroughly for a few years until the roots are well established (Crockett, 1972; Robertson, 1977). Furthermore, *C. kentukea* is hardier and has more ornamental characteristics than the three other *Cladrastis* sp. (Sand, 1992). Generally, yellowwood can be found in rich, well-drained limestone soils in river valleys and slopes and ridges along streams (Dirr, 1998).

Graves and van de Poll (1992) reported and confirmed earlier findings (Allen and Allen, 1981) that among Papilionoideae subfamily, the species of the genus *Cladrastis* are some of the few trees that do not nodulate with bacteria. Although yellowwood is a leguminous tree, growers of this species cannot rely on symbioses with rhizobia to provide plants with nitrogen (Graves and van de Poll, 1992).

Yellowwood can be found in zones four to eight. Its native habitat is considered to be southeastern United States, including North Carolina, Kentucky and Tennessee, but is not very common anywhere (Dirr, 1998) (Figure 2.4.). According to Robertson (1977), yellowwood is endemic in the eastern United States, occurring from Brown County in Ohio, and Brown County, Indiana west through southern Illinois to southwestern Missouri, northern and central

Figure 2.4. *Cladrastis kentukea* range map. (U.S. Department of the Interior & U.S. Geological Survey, 2004)

Arkansas, and extreme eastern Oklahoma, south through central Tennessee to central and southern Alabama, and east to the mountains of eastern Tennessee, North Carolina, and extreme northern Georgia (Robertson, 1977).

2.14 Cladrastis kentukea Insect and Disease Problems

Generally, yellowwood trees are not afflicted with any major diseases. However, yellowwood is intolerant to excess water and sensitive to misapplied phenoxy pesticides (Sinclair et al., 1987). Several diseases caused by *Botryosphaeria dothidea a* (Moug.: Fr.) Ces.&DeNot. (syn. *Botryosphaeria ribis*, *B. berengiana*), *Phyllactinia guttata* (Wallr.:Fr.) Lev., *Fomitopsis spraguei* (Berk. & M. A. Curtis) R. L. Gilbertson and Ryvarden, *Oxyporus latemarginatus* (Durieu & Mont.) Donk (Farr et al., 1989), V*erticillium albo-atrum* Reinke and Berthier (Dirr, 1998; Farr et al., 1989) and yellowwood anthracnose, caused by *Discula* and *Gloeosporium sp.* (Hong et al., 2003; University of Kentucky - Cooperative Extension Service, 2000) have been associated with this ornamental tree.

Botryosphaeria dothide (Botryosphaeria canker - dieback), is a non-specialized, rather opportunistic pathogen, infecting more than 100 genera, and is wide spread in temperate and tropical regions around the world (Sinclair et al., 1987). Abiotic diseases often predispose plants to fungal disease, such as *Botryosphaeria sp*. (Windham and Windham, 2003) as well as other causal agents. Although most canker causing fungi are Ascomycetes, only a few of

them, including *B. dothidea,* produce their sexual stage regularly (Agrios, 1997) . *Botryosphaeria dothidea* is generally associated with annual or perennial cankers and dieback in wounded plants but can also be found in plants suffering from drought, freezing, or defoliation, as well as in trees pruned the previous year (Sinclair et al., 1987). Nevertheless, diagnosis of disease caused by *B. dothidea* requires recognition of its conidial (*Dothiorella*) state, which is characterized by black pycnidial stromata that differentiates beneath the surface of killed bark and breaks through at maturity (Sinclair et al., 1987).

Phyllactinia guttata (syn. *Phyllactinia corylea* (Pers.) P. Karst.) commonly known as powdery mildew, is found on alders and numerous other hardwood (Daughtrey et al., 2003), including yellowwood. Preston (1945) and an anonymous author (1960) published the first reports detailing *P. guttata* on yellowwood (Farr et al., 1989). Powdery mildew in general can cause reduction in aesthetic value affecting the market value of the plant. It can also lessen flower production and quality, lower photosynthetic efficiency that results in reduced plant growth, increase likelihood of winter injury, due to the physiological weakening of the plants, but rarely causes death of the plant (Pataky, 1987).

Phyllactinia guttata is easily recognized because of its characteristic cleistothecia with bulbous-based appendages. These appendages are moisture sensitive and have a very significant and unique function in dispersal of its spores. When mature, these appendages press down on a substrate, breaking the ascocarp away and releasing it into the wind (Daughtrey et al., 2003). The fungus overwinters in an upside down position and in spring, the ascoma splits

open around its equator reveling asci in its “lid” in the perfect position to discharge infective spores into the air. Other powdery mildews have less complex appendages and typically split open under the pressure of asci against the upper surface (Daughtrey et al., 2003).

Fomitopsis spraguei as well as *Oxyporus latemarginatus* cause wood rot (The American Phytopathological Society, 2004). However, they are rarely found on yellowwood trees and there is little available information about these two pathogens.

Verticillium albo-atrum is a soil-borne pathogen that causes Verticillium wilt. It may remain viable as dormant mycelium or black, microsclerotia in the soil, where it overwinters for 25 years or longer, or by surviving between cultivated crops on susceptible weeds. Species of this genus produce slender conidiophores bearing whorled (verticillate) phialides that give rise to small, hyaline, one celled conidia (Alexopoulos et al., 1996). Symptoms of infected plants include dropping of outer and older leaves. Diseased leaves usually wilt, turn dry, and become reddish yellow or dark brown at the margins and between the veins (Babadoost, 2001). This process leads to stunted and overall poor growth as well as development of dwarfed roots with blackened tips. If new leaves do form, they are often stunted and may wilt and curl up along the mid vein (Babadoost, 2001).

Although very uncommon for yellowwood, anthracnose has also been observed on yellowwood in Louisville, Versailles, (University of Kentucky - Cooperative Extension Service, 2000) and Ohio. According to Cooperative

Extension Service in Kentucky, yellowwood anthracnose is caused by a *Discula sp*. (Chatfield et al., 2000). Contrary to this finding, (Hong et al., 2003) at Virginia Cooperative Extension stated that *Gloeosporium sp.* caused anthracnose on yellowwood trees.

2.15. *Cladrastis kentukea Propagation Methods*

This tree is rather difficult to find in nurseries and, when available, it is usually in smaller and very limited quantities (Robertson, 1977). Yellowwood is difficult to propagate by conventional vegetative methods, therefore commercial propagation of yellowwood relies on seed, which requires scarification and stratification (Dirr, 1998). Stratification usually takes up to three months at 4-5ºC, however, prior to this process, seeds should be covered with hot water (88ºC), and allowed to set overnight (Robertson, 1977). Also, the seed supply may be limited from year to year due to sporadic blooming cycles of yellowwood, making propagation practices even harder. Yellowwood are also propagated by root cuttings, however, this practice is relatively rare (Robertson, 1977).

2.16 Cladrastis kentukea Tissue Culture

A major advantage of micropropagation in angiosperm trees is the wide range of explants available, such as seeds, seedling parts, leaves, shoots and buds from both mature and juvenile trees, floral parts and in some cases even

roots (Khurana et al., 2003). Propagation by means of tissue culture can enhance plant efficiency and provide year-round production with high quality plants. Tissue culture also offers rapid mass propagation methods, genetic uniformity of a clone as well as an alternative technique of propagation for this native species. However, many difficulties, including excessive phenolic-like compounds associated with culture initiation, and reluctant nature of shoot and root formation related to the ontogenetic stage of maturity inherent in a perennial woody crop, are encountered during micropropagation of woody legumes (Trigiano et al., 1992b). This can generally be applied to many woody species, including yellowwood because of its limited information provided about propagation of this species.

Since it ensures a high degree of clonal fidelity, axillary bud proliferation is often used tool for propagation of ornamental crops and preferred by commercial tissue culture laboratories (Hu and Wang, 1983). Yellowwood, with its appealing attributes and characteristics has ornamental qualities and the potential for wider geographical distribution and availability. Nonetheless, its value in nursery and landscape production may be limited by the lack of an efficient method of propagation (Weaver, 1990).

Although axillary bud proliferation and somatic embryogenesis have been accomplished for some trees in the Fabaceae, requirements for *in vitro* propagation of legumes are species dependent (Weaver, 1990). The only tissue culture propagation method for yellowwood was reported by Weaver and Trigiano (1991). They indicated that yellowwood could be regenerated through

axillary bud proliferation (Weaver, 1990) and somatic embryogenesis from immature zygotic embryos (Weaver and Trigiano, 1991). Somatic embryos were formed directly at the base of immature cotyledons, resembling a similar somatic embryogenesis procedure for two other legume trees – *Cercis canadensis* L. (Geneve and Kester, 1990) and *Robinia pseudoacacia* L. (Merkle and Wiecko, 1989).

2.2 Materials and Methods

2.21 Cladrastis kentukea Explant Preparation

All yellowwood plant material originated from a single, 35-40 year old tree located on the University of Tennessee Agricultural campus in Knoxville, TN, eliminating potential genotype effects in the experiments. Axillary buds were removed from elongated branchlets during the last three weeks of April 2003 – 17th, 24th and 29th, before anthesis or flowering. After the lamina and most of the petiole were eliminated, nodal segments were removed one centimeter above and below enlarged clasping petiole bases enclosing the axillary buds. Stem segments were dipped in 95% ethanol, flamed for 10 seconds, and then surface sterilized for eight to ten minutes in 20% v/v commercial bleach solution (Clorox®) amended with 0.1%Triton X-100 with constant stirring. Explants were rinsed three times with sterile distilled water. After surface sterilization procedures, the cut ends exposed to the bleach were removed, and axillary buds

were placed in 20x150 mm glass culture tubes. The two basal media, Murashige and Skoog (MS) (Murashige and Skoog, 1962), supplemented with 30g/l sucrose, 0.1g myo-inositol and vitamins (pyrodixine HCl 0.5mg/l, thiamine HCl 1mg/l, nicotinic acid 0.5mg/l and L-glycine 2 mg/l) (Appendix 1) and Woody Plant Medium (WPM) (Lloyd and McCown, 1980) supplemented with 25g/l sucrose, 0.1g myo-inositol and vitamins (pyrodixine HCl 0.5mg/l, thiamine HCl 1mg/l, nicotinic acid 0.5mg/l and L-glycine 2 mg/l) (Appendix 2), were amended with either 0, 1, 2 or 4 µM 6–benzylaminopurine (BA). The pH of all media was adjusted to 5.8 with 0.1 N KOH prior to autoclaving (25 minutes at 121°C). Cultures were incubated in incubators and later in the study were maintained in the growth room at 23ºC with 55 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ light intensity for 16/8 light-dark photoperiod. After the first cycle, all cultures were transferred to vessels for plant tissue culture (175ml capacity, 98.5mm high), with magenta B caps made by Sigma (St. Louis, MO) and placed onto fresh medium every 4-5 weeks. After 5 cycles, all explants were transferred, regardless of initial BA concentration, to medium containing 2 µM BA, following earlier transfer regime of 4-5 weeks and same light requirements. Basal callus was excised after each transfer to fresh medium, and terminal shoot tips were removed from explants to reduce apical dominance and stimulate axillary shoot growth from lateral buds. Explants were acclimatized to in vitro conditions for approximately nine to ten culture cycles before the microshoots were used for rooting experiments.

2.22 Experimental Procedure

Thirty-nine weeks after initial culture, three centimeters long elongated shoots, were harvested from proliferating cultures and transferred to Magenta GA-7 vessels containing half-strength Murashige and Skoog (MS) medium supplemented with 6g/L agar. Each vessel contained about 100 ml of the experimental root-inducing medium. Indole-3-butyric acid (IBA) was filter-sterilized using a 0.22 µm syringe filter and added to the medium after the medium had cooled, but not hardened, at the following concentrations: 0, 3, 30, 100, and 300 µM. After an initial pulse treatment of three days, explants were placed in 17x100 mm plastic, disposable culture tubes with plastic snap-on caps (Fisher, Atlanta, GA), containing about 5 ml half-strength MS medium without plant growth regulators. The cultures were placed in racks in a growth room at 21-23°C for four weeks with $\mu mol \cdot m^{-2} \cdot sec^{-1}$ light intensity for 16/8 light-dark photoperiod. Proliferating masses were placed on fresh medium with 2 µM BA and were used again for the rooting projects. Over the time, WPM medium was unsuitable for yellowwood *in vitro* production, resulting in stunted growth, necrosis, yellowing and overall poor performance of the plants, and were excluded from the study.

2.23 Experimental Design

The experimental design was a randomized complete block design (RCBD) with four explants per each Magenta vessel and six replications for each treatment. The experimental unit consisted of one microshoot per culture tube, resulting in 120 explants. In randomized complete block designs, treatments are grouped into blocks that contain at least one replicate from each treatment. Experimental units are randomized within blocks, each employing a separate randomization scheme. This design was selected because it minimizes variability within a block while maximizing variability among blocks (Compton, 2000). After four weeks, data were collected including the number of rooted microshoots, number of roots per rooted microshoot, length of each root, number of damaged meristems, presence or absence of lenticels, and number of roots. Data were analyzed using analysis of variance (ANOVA) and mean separation was done on the transformed data using the method of least significant differences (LSD) at P=0.05 (SAS Institute, 1996).

2.24 Acclimatization of Microshoots

After four weeks on rooting medium, whole plants were washed with tap water to remove agar and placed in 5.5 cm in diameter round plastic pots containing autoclaved, general purpose growing medium, Pro-Mix ‘BX’ (Premier Horticulture Ltd., Pennsylvania) (Appendix 3) (Figure 2.5.). Rooted microshoots

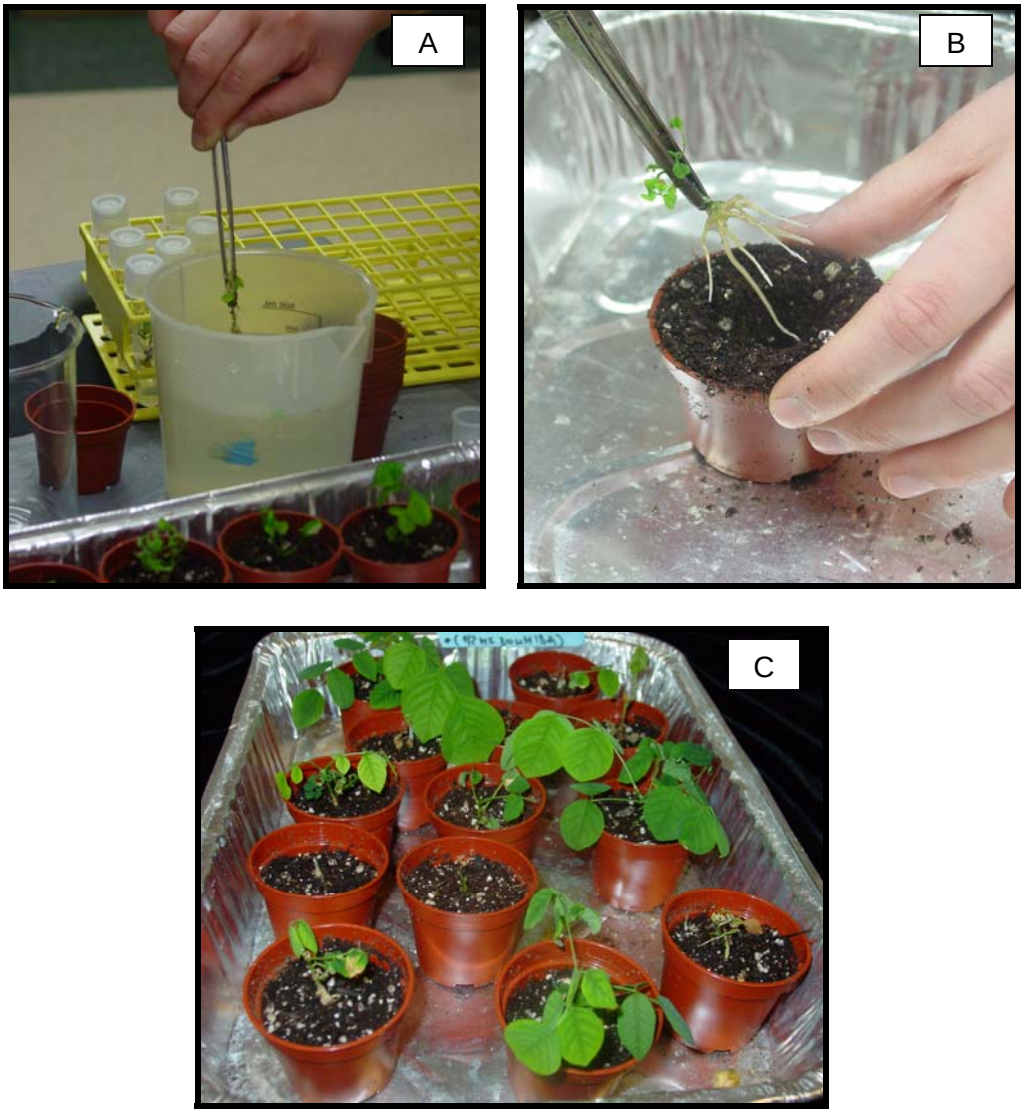


Figure 2.5. Acclimatization procedure for *Cladrastis kentukea* rooted explants. (A) Washing of plants with tap water to remove agar; (B) placing rooted microshoots gently into plastic pots containing general purpose growing medium, Pro-Mix 'BX', to avoid breaking and possible root damage; and (C) rooted microshoots in aluminum trays after few weeks in the growth chamber with lids gradually removed to acclimatize plants.

were placed gently in the media to avoid breaking and possible damaging the roots. Pots with rooted microshoots were placed in 323x228x46 mm Hefty cake aluminum pans with plastic lids (Pectiv Corporation, Lake Forest, IL) (Figure 2.5.). Rooted yellowwood plants were then placed in the growth room at 21-23°C with 55 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ light intensity for 16/8 light-dark photoperiod for acclimatization. Aluminum pans with plastic lids were used as a humidity chamber in which pots with entire plants were placed to maintain high relative humidity level. A few holes, 0.5cm in diameter, were punched in the lid and the number of holes gradually increased over the next few weeks, allowing plants to adapt to a non-sterile, ambient environment. Plants were initially watered with tap water every other day or when needed after the lids were removed completely, and thereafter with 10% MS basal salts without any sucrose when needed. Washed plants were easily adapted from transfer to growing media and within four weeks were acclimated to ambient conditions of the growth room (Figure 2.6.). In August of 2004, yellowwood plants were transferred to plastic, round pots (12x12cm) amended with Sunshine Professional Peat –Lite LC #1 soil mixture (Sun Gro Horticulture Canada Ltd, Canada) (Appendix 4) and fertilized with Miracle Grow, general-purpose plant food (1.5 tablespoons/ 1 gallon water) (Figure 2.7.). In November 2004, plants were moved from the greenhouse environment to cold frames, and subsequently moved to an overwintering house.

Figure 2.6. Acclimatized *C. kentukea* plantlets that were exposed to MS media supplemented with 30 µM IBA (upper left), 100 µM IBA (upper right) and 300 µM IBA (bottom).

Figure 2.7. Acclimatized *Cladrastis kentukea* plants in the greenhouse environment, placed in 12x12 plastic pots containing Sunshine LC-1 soil mixture. Note expended leaves and elongated shoots.

2.25 Results and Discussion

Pulse treatments for root induction proved to be the most successful with microshoots exposed to the highest concentrations of IBA. In total, 18 out of 24 explants rooted when exposed to 300 µM IBA for three days, resulting in 75% rooted microshoots (Figure 2.8.) (Table 1). Fifty-four and 46 percent of the microshoots exposed to 100 and 30 µM IBA rooted, representing a total of 13 and 11 microshoots rooted retrospectively (Table 1) (Figure 2.8.). Only 4% of the microshoots rooted when exposed to 3 µM IBA for three days, representing a single plant (Figure 2.8.), and none of the control (without IBA) microshoots rooted (Table 1). There was no significant difference between the numbers of microshoots exposed to 30 and 100 µM IBA treatments (Figure 2.9.). Significant differences ($P<0.05$) were observed with the explants exposed to 300µM of IBA compared to other treatments (Figure 2.9.). Although this treatment induced the most root formation, there was significantly higher (F=4.11; df= 2, 35; P<0.025) terminal meristem abortion compared to the microshoots treated with 100 and 30 µM IBA (Table 1) (Figure 2.10.). When exposed to concentrations of 100 and 30 µM of IBA, the number of microshoots that rooted did not significantly differ -- 54% and 46% of microshoots rooted, respectively. Also, when comparing meristem tip damage, these two treatments showed no significant difference between each other (F=0.08; df=1, 35; P=0.7756). However, there was significantly less terminal damage observed with microshoots exposed to 100 µM vs. 300, as well as to 30 vs. 300 µM of IBA

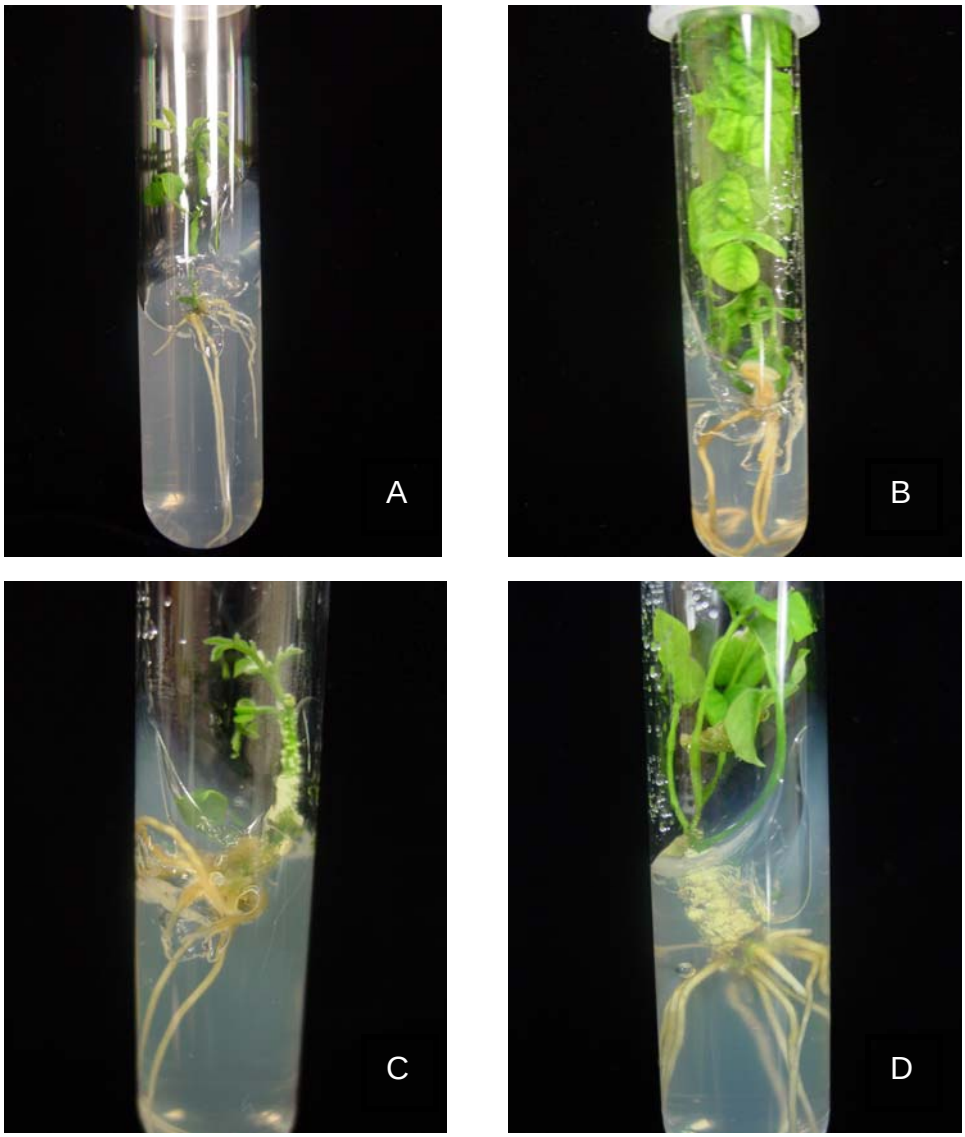


Figure 2.8. *Cladrastis. kentukea* rooted microshoots exposed to MS amended with 3 (A), 30 (B), 100 (C) and 300 µM IBA (D). Rooted microshoots exposed to 3, 30 and 100 µM IBA exhibit long, brown and thick roots. Also, the 3 µM IBA treatment was represented by a single rooted plant. Note abscised leaves, stunted growth and presence of lenticels in explant exposed to 100 µM IBA.

Table 1. Effect of IBA on rooting of *Cladrastis kentukea* microshoots after four weeks on Murashige and Skoog (MS) supplemented with IBA. Values within a column with different superscript are significantly different ($P < 0.05$). Data includes means of all explants treated with IBA. Statistical differences were assessed by using method of least significant differences (LSD) at $P=0.05$.

IBA concentration (µM)	Number of rooted explants/total number of explants	Mean percentage of rooted explants	Standard error	Mean number of roots/rooted microshoot	Mean number of total root length/rooted microshoots (mm)	Mean shoot damage/rooted microshoot
0	0/24	0^{c}	0	0^{a}	0^{a}	0^{b}
3	1/24	4.1^{c}	0.04	5^{a}	122.4^{a}	1^{b}
30	11/24	45.8^{b}	0.10	4.45^{a}	108.05^{a}	1.72^{ab}
100	13/24	54.1^{b}	0.10	4.46^{a}	96.39^{a}	1.61^{ab}
300	18/24	75.0^{a}	0.09	6.61^{a}	104.11^{a}	2.44^{a}

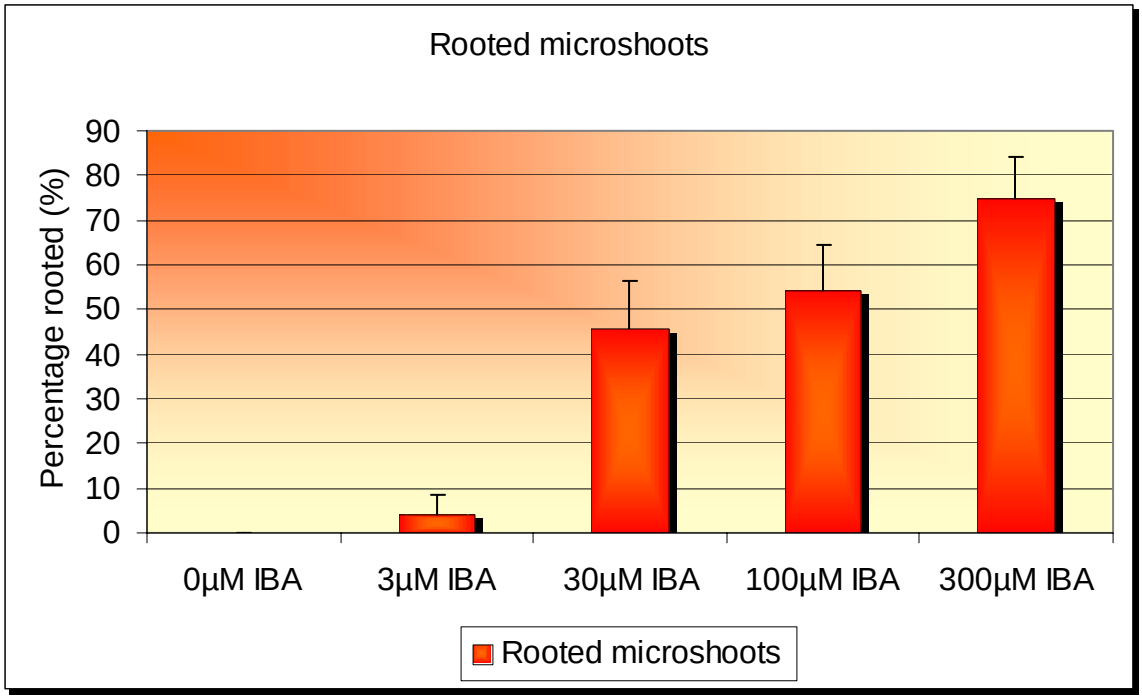


Figure 2.9. Effect of IBA pulse treatments on root formation on *Cladrastis kentukea* microshoots. Vertical bars represent standard errors.

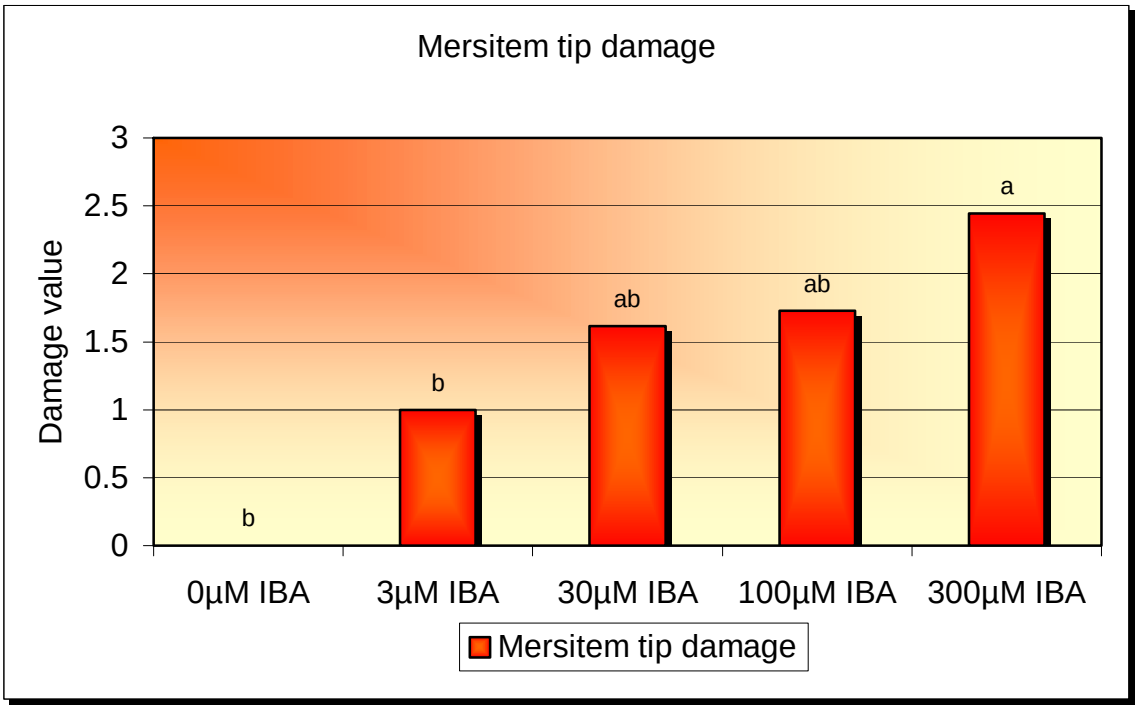


Figure 2.10. Effects of IBA on meristem tip damage of *Cladrastis kentukea* microshoots. Mean separation was done on the transformed data using the method of least significant differences (LSD) at *P*=0.05.

(F=7.06; df=1, 35; *P*<0.0118; and F=4.78; df=1, 35; P<0.0356; respectively) (Figure 2.10.). Interestingly, no statistical differences were found between the numbers of roots and total root length among all treatments (Table 1). Nonetheless, lenticels were present on all microshoots that rooted (Figure 2.11.). This suggests lenticel cambial activity can possibly indicate the ability of selected microshoots to produce roots. Furthermore, better balance between exposure and timing of auxin treatments for rooting is critical and will require further studies. When comparing means among all rooted explants, which exclude control, no statistical differences (*P*<0.2391 and *P*<0.9755 retrospectively*)* were found for the number of rooted microshoots and total root length. However, significant differences were observed with terminal tip damage among rooted microshoots (*F*=2.97; df=3, 35; *P*< 0.0451).

Pulse treatment for increased rooting, however, proved to be quite successful with different plant species. Pulsing microcuttings with high auxin was employed in regeneration of chestnut shoots, which required only a short 24 h pulse on IBA (Sanchez et al., 1997). Bidwell et al. (2001) reported that the exposure time of shoots to auxins was critical for successful *in vitro* rooting of nickel hyperaccumulator, *Hybanthus floribundus.* They concluded 24 h exposure to 100 µM IBA followed by transfer to hormone free medium was the most successful treatment for root induction.

The induction of root morphogenesis to obtain whole plants from legumes has been conventionally approached by means of studies of different auxins at different concentrations (Fratini and Ruiz, 2003). IBA, a synthetic auxin, was

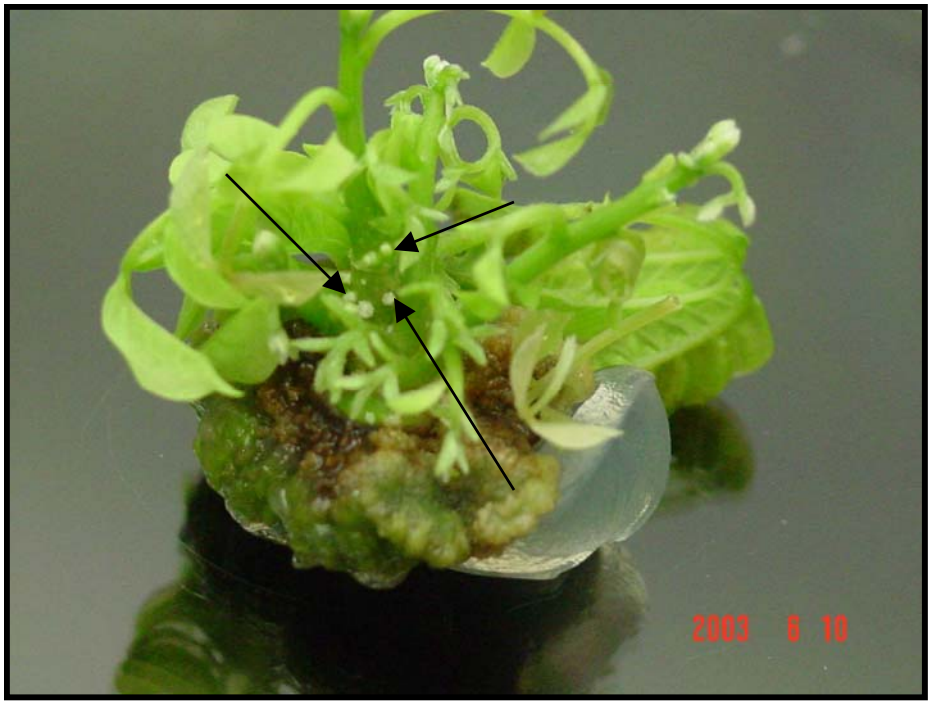


Figure 2.11. *Cladrastis kentukea* microshoot exhibiting lenticels (arrows). All rooted microshoots had lenticels, suggesting its significant role as an indicator of the microshoot ability to form roots.

reported to induce adventitious roots on a wide range of different species (George, 1993). Therefore, it was chosen to induce rooting in yellowwood experiments based on previously reported yellowwood research (Weaver and Trigiano, 1991), as well as other woody species.

Rout and Das (1993) reported that out of three auxins tested (IAA, NAA and IBA), only IBA promoted rooting. IBA is often used in higher concentrations for pulse treatments, or lower concentrations for extended periods of time. Preceding yellowwood research did not employ pulse treatment for yellowwood root induction. They reported the best rooting occurred on half strength MS medium supplemented with 4.9 µM IBA and cultured in the dark (Trigiano et al., 1992b; Weaver, 1990). Monteuuis and Bon (2001) also found the proportion of rooted microshoots was significantly enhanced by darkness compared with 16/8 standard lighting conditions. They reported increased rooting of *A. mangium* microshoots exposed in darkness to 4 and 6 µM IAA and to the lesser extent IBA. More precisely, microshoots that were cultured in darkness formed more, but shorter, adventitious roots than those exposed to light treatment (Monteuuis and Bon, 2001).

One of the principle problems encountered with tissue culture propagation of different leguminous plant species is the difficulty of excised shoots to produce roots (Davis and Keathley, 1987). Difficulties with successful root formation were observed with other leguminous genera such as *Cercis* (Bennett, 1987) and *Robinia* (Davis and Keathley, 1987). However, frequent subculturing proved to be successful for rooting of *Cercis canadensis* var. alba (Eastern redbud)

(Yusnita et al., 1990), *Cercis yunnanensis* (Yunnan redbud) (Cheong and Pooler, 2003) and *Acacia mangium* (Mangium) (Monteuuis and Bon, 2001) microcuttings, as well as *C. kentukea*.

Trigiano et al. (1992b) found that serial subculturing had a rejuvenating effect and could be used as an alternative strategy for the successful recovery of plants from difficult-to-propagate species where ontogenetic age is potentially the limiting factor (Hackett, 1985). However, the legumes of tropical origin were not found to be problematic to reproduce through means of tissue culture and rooting did not represent a significant problem (Trigiano et al., 1992b).

Optimum cultural concentrations of cytokinins necessary for axillary bud multiplication may be specific for each system and different depending on the leguminous tree species. In a previous study (Weaver, 1990), the most consistent bud formation, elongation, and number of harvested yellowwood shoots over the six months culturing period occurred on medium supplemented with either 2.2 or 4.4 µM BA (Trigiano et al., 1992b), although 2.2 µM BA initially produced the most harvestable shoots (Weaver, 1990). Similar findings were observed in *Madhuca longifolia* var. latifolia (Mahua) (Rout and Das, 1993), *Acacia mangium* (Mangium) (Monteuuis and Bon, 2001) and *Acacia catecha* (Kour et al., 1998). Both *Acacia species* required lower concentrations of IBA and NAA, respectively, for multiplication media. *Dalbergia sissoo,* probably the most closely related arborescent genus to yellowwood, produced an average of 10-15 shoots per cotyledonary node on media containing 4.4 µM BA and 0.54 µM IAA (Suwal et al., 1988).

After a single culture cycle on two different media, WPM and MS, yellowwood axillary buds showed differences in shoot proliferation and elongation, as well as callus formation. When the effects of these two media on proliferation were compared using 1 or 2 µM BA, the difference among the treatments was evident (Figure 2.12.). Microshoots exposed to WPM media appeared stunted, with compacted growth and reduced numbers of microshoots. When they were compared after two cycles (Figure 2.13. and Figure 2.14.), MS proliferation media supplemented with 2 µM BA seemed the best for inducing yellowwood microshoot proliferation, stimulating better overall growth and producing the best and most consistent number of shoots. This finding was further confirmed throughout the experiment when after five cycles, WPM media was excluded from further studies due to poor performance.

During the shoot proliferation process, terminal microshoots of all cultures were eliminated in order to stimulate axillary shoot growth from lateral buds and reduce apical dominance. Differences between the control (0 µM) and other treatments were apparent (Figure 2.13. and Figure 2.14.). Also, increased BA concentration over time resulted in a decreased mean number of shoots per explant or had no effect at all on forming microshoots. Higher concentrations of BA also negatively influenced the number of shoots produced by cultures of C. *yunnanensis* (Cheong and Pooler, 2003) and *Dalbergia sissoo* (Singh et al., 2002). Explants performed visibly stunted growth, increased shoot tip necrosis, and suppressed shoot elongation (Figure 2.13. and Figure 2.14.). The same observations were noticed in an earlier yellowwood study. Weaver (1990)

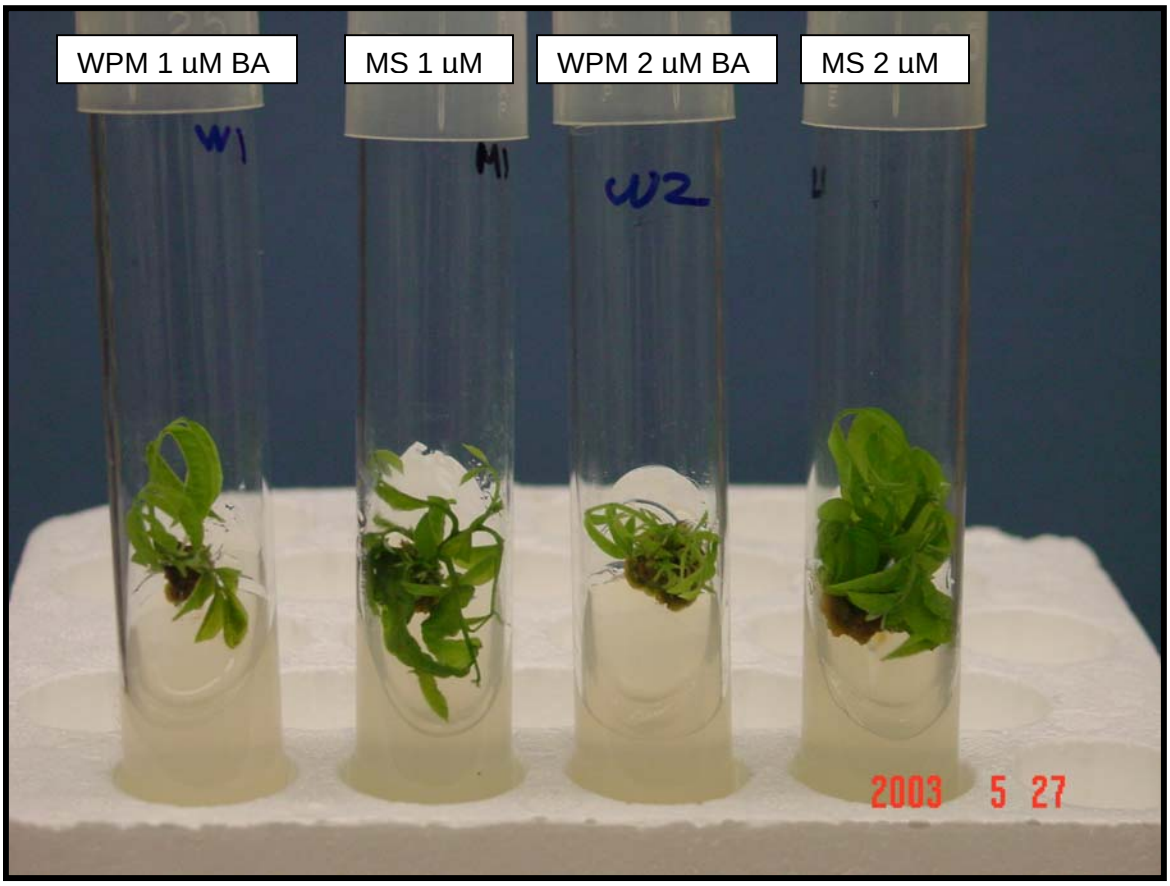


Figure 2.12. *Cladrastis kentukea* axillary buds placed in 20x150 mm glass culture tubes after the first cycle. Compared Woody Plant Medium (WPM) Murashige and Skoog (MS) amended with either 1 or 2 µM BA. Note the differences among media and BA concentrations. WPM 2 µM BA after the first cycle appeared stunted with compacted growth and reduced numbers of microshoots. Contrary to this finding, MS amended with either 1 or 2 µM BA induced better microshoot proliferation and overall better growth of yellowwood microshoots.

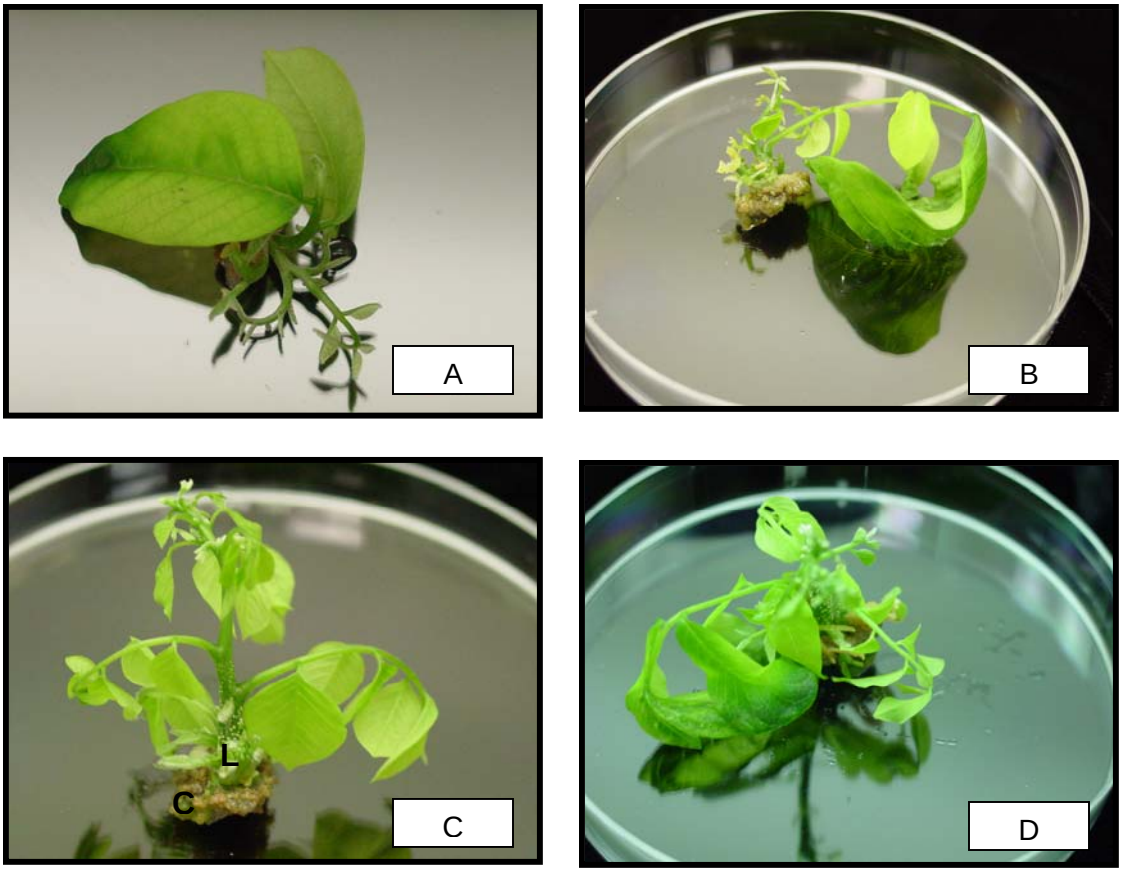


Figure 2.13. *Cladrastis kentukea* microshoots after two cycles on Murashige and Skoog (MS) proliferation media amended with 0, 1, 2 and 4 µM BA (A, B, C and D) respectively. A. Note the difference between 0 µM and other treatments; B. Enlarged leaves and poor shoot development with microshoots exposed to lower BA concentrations; C. Increased shoot elongation with explants exposed to 2 µM BA, which throughout the experimental trials, proved to be the best suited for rooting experiments; also, lenticels (L) and callus (C) formation are noticeable; D. Microshoots expressed visibly stunted growth and deformed leaf formation when exposed to higher BA concentrations.

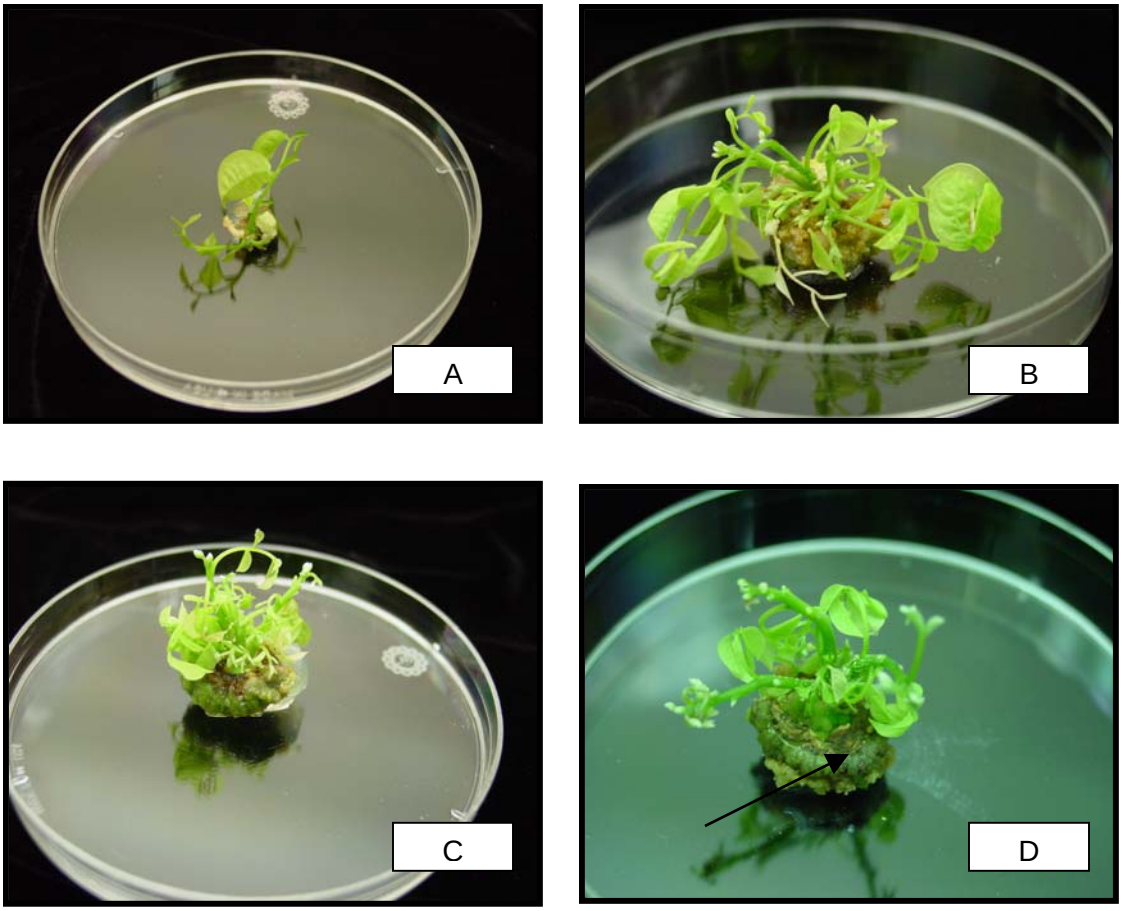


Figure 2.14. *Cladrastis kentukea* microshoots after two cycles on Woody Plant Medium (WPM) proliferation media amended with 0, 1, 2 and 4 µM BA (A, B, C and D) respectively. A. Note the difference between 0 µM and other treatments; B. Extensive but relatively short microshoots proliferation and callus formation; C. Note the reduction in length of shoots. D. Extensive callus formation (arrow) and obvious stunted growth. Microshoots placed on MS medium exposed to the same concentration of BA appeared similarly.

noted the average length of shoots from axillary buds decreased with increasing BA concentrations. For example, shoot elongation at 22 µM BA was slow and suppressed as compared to other treatments used in the study (Weaver, 1990). Although these high BA treatments formed the greatest number of buds, the shoot did not reach a harvestable length of a minimum 0.5 cm (Weaver, 1990).

Similarly to preceding yellowwood research, decline in overall performance of cultures after the 12th cycle resulted in decreased vitality and reduced shoot production. Contrary to this finding, Suwal et al. (1988) and Monteuuis and Bon (2001) successfully maintained their *D. sissoo* and *Acacia mangium* cultures for two and three years, respectively, without a loss of multiplication potential. Further research is required for better understanding of optimum culture requirements as well as improved protocol for maintaining proliferating cultures for longer period of time.

In the conducted research, MS medium resulted in better proliferation of yellowwood axillary buds than WPM. Therefore, after the fifth cycle, WPM cultures were eliminated from further research experiments. Contrary to this finding, *R. pseudoacacia* L. (black locust) cultures responded equally well on both WPM and MS media (Merkle and Wiecko, 1989). Several factors such as fluctuations in temperature in the growth room as well as electricity outage for extended period of time contributed to yellowwood decline. However, further research is necessary to confirm this conjecture.

The developmental or physiological stage of the explant strongly influenced the ability of an explant to respond morphogenetically (Williams and

Maheswaran, 1986). Weaver and Trigiano (1991) found the lack of conversion of yellowwood somatic embryos to plantlets might be due to physiological immaturity or anatomical abnormalities (Ammirato, 1987) as it was seen with the abnormal shoot apex development of *C. canadensis* L. (Trigiano et al., 1988). Hackett (1985) states the success of either shoot or root formation *in vitro* is often related to the maturity of the donor plant. Also, the type of explant used in shoot multiplication of Chinese redbud (Cheong and Pooler, 2003) as well as age and the length of media exposure time for Black locust (Merkle and Wiecko, 1989) had a significant effect on the number of shoots produced.

Another interesting observation was found in a cotton study in which Hazra et al. (2000) confirmed the prior results of Agrawal et al.(1997). In this study, more multiple shoots were obtained from explants excised from seedling grown in conical flasks compared to those grown in tissue culture tubes. This observation of the positive influence of larger culture vessel on shoot proliferation was consistent with earlier findings related to five woody species (McClelland and Smith, 1990).

This research indicates yellowwood is capable of regeneration by axillary bud proliferation. Murashige and Skoog basal proliferation medium amended with 2 µM BA resulted in the most consistent number of shoots throughout the experiment. The 300 µM IBA yielded in most rooted explants, however, this treatment also resulted in significantly higher terminal meristem abortion compared to other treatments. Only four yellowwood plants survived, suggesting better acclimatization practices for this species are needed. This study

presented protocols and established methods for yellowwood *in vitro* regeneration, providing a new approach to mass production of this valuable ornamental tree for the southeast region of the United States. Further research is necessary to improve acclimatization practices and reduce meristem tip damage, thus producing more vigorous plants.

Chapter Three

Cornus kousa

3.1 Literature Review for *Cornus kousa*

3.11 Cornus kousa (Buerger ex Miq) Hance Introduction

Dogwoods belong to the family Cornaceae, which is represented by a single genus, *Cornus* L. The genus encompasses approximately 65 distinct species (Eyde, 1988) of small, deciduous or evergreen trees (Toogood, 1999). Dogwood species, including *C. kousa* (Buerger ex Miq.) Hance, are predominantly cultivated for their ornamental characteristics, such as showy bracts, attractive seasonal foliage, fruit and twig color, but also have other minor uses (Trigiano et al., 1992a). *Cornus kousa* is commonly known as Japanese Dogwood or Kousa Dogwood and is a commercially important cultivated species. The plant is native to Japan, Korea and China and was introduced to the United States in 1875 (Dirr, 1998). Bark of some cultivars is attractive, and in such cases, lower branches are often thinned to show off bark characteristics. Although young trees exhibit only limited bark exfoliation, the tree shows its true bark character as it gets older (Gilman and Watson, 1993). Planting a Kousa dogwood can extend the spring flowering season several weeks since it blooms about a month after flowering dogwood (*C. florida* L).

3.12 Cornus kousa Morphology

Cornus kousa has simple, opposite, elliptic to ovate leaves that are usually two to four inches long (Dirr, 1998) with almost blue-green coloration in summer. Vegetative buds are valvate and appresed, brownish to black and usually longer than those in *C. florida* (Dirr, 1998). Flower buds, formed at the end of the stem are fattened and globose at the base with two valvate, silky, appressed pubescent bud scales forming a sharp apex (Dirr, 1998).

Flowers usually appear in May or June. Blooms have four-pointed flower bracts about as large as those of flowering dogwood, however, the foliage develops before the bracts appear. Also, the long lasting bracts are located above the leaves on long flower stems (Witte et al., 2000).

Cornus kousa fruit resemble raspberries in appearance. It is an edible fleshy aggregate fruit (Griffiths, 1994) borne on a globose syncarp (Dirr, 1998). The aggregate fruits are rosy-red balls, the size of a nickel or larger, and contain several seeds embedded in pulpy orange flesh (Witte et al., 2000). Fruits are borne on two to two and a half inches long pendulous stalk (Dirr, 1998) and appears in late July throughout October, which gives a beautiful coloration and contrast to the green foliage and adds additional landscape value for this species. Kousa fruits are very showy and attractive for birds however, they do not represent significant litter problem (Gilman and Watson, 1993).

Young *C. kousa* plants are vase-shaped, but with age, they form a rounded appearance with a distinct stratified branching pattern and very strong

horizontal lines. Growth rate is relatively slow to medium in the early stages of development, becoming arched and layered with age, reaching about 6-9m when mature. The tree is considered very ornamental, especially when in flower in late spring (Figure 3.1.), but also when heavily laden with fruit and with its fall color. During the fall season, foliage changes to reddish purple or scarlet and usually persists for three to five weeks. The bark exfoliates with age, forming a mottled mosaic of gray, tan and rich brown colors (Dirr, 1998).

3.13 Cornus kousa Culture

Cornus kousa prefers a rich well-drained soil that receives regular rainfall throughout the growing season (Witte et al., 2000) and a position that is at least partially sunny. However, it will succeed in almost any soil of good or moderate fertility, ranging from acidic to slightly alkaline and still flowers abundantly in light to moderate shade. Optimum soil for kousa dogwood growth is slightly acid and contains sufficient organic matter to prevent the soil from drying out quickly during the hot summer periods (Witte et al., 2000). It grows well in heavy clay soils, however it does poorly in shallow, chalky soils. Kousa dogwoods can be found in both sun and shade. *Cornus kousa* is commonly planted in the USDA hardiness zones five to eight (Dirr, 1998; Gilman and Watson, 1993) with a wide potential planting range (Figure 3.2.).

Kousa dogwoods are more tolerant of dry conditions than the native flowering dogwood (Malinoski and Clement, 2003). *Cornus kousa* leaves remain

Figure 3.1. *Cornus kousa* blooms (courtesy of Mark Windham, University of Tennessee).

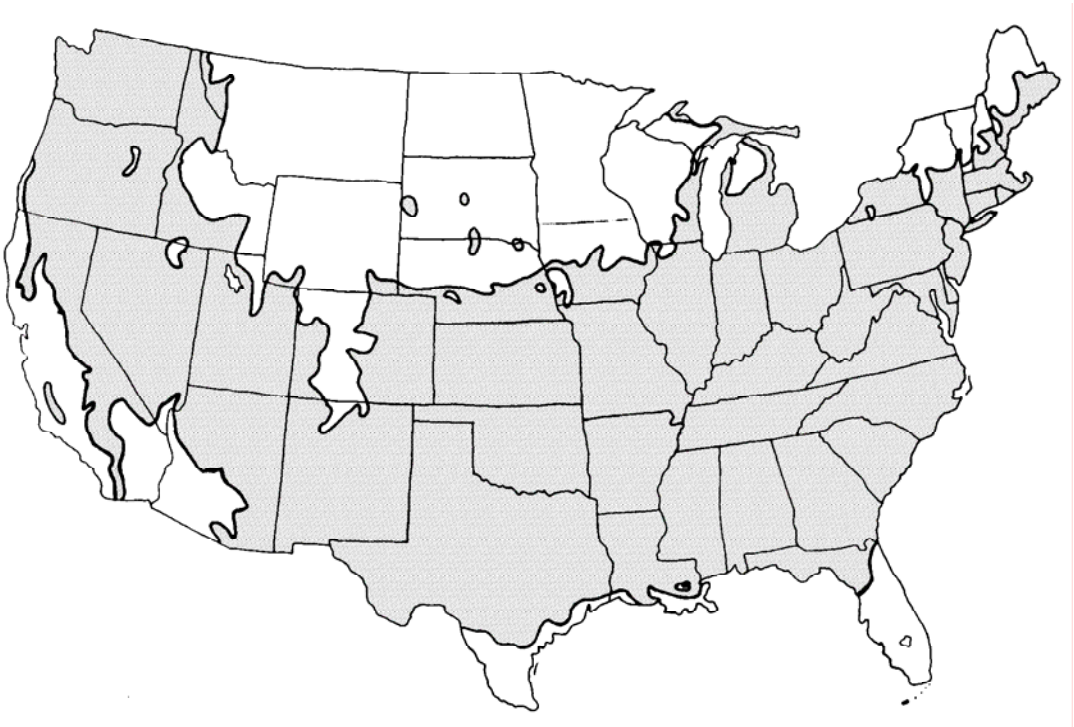


Figure 3.2. Shaded area represents potential planting range of *Cornus kousa*. (Adapted from Gilman and Watson, 1993)

on the tree, but curl with draught and heat, exposing the lighter, somewhat muddy-colored abaxial surface, whereas leaves on C*. florida* tend to drop in response to draught stress (Auge et al., 2002).

Kousa dogwoods are not typically susceptible to either dogwood anthracnose or powdery mildew as is flowering dogwood. Kousa dogwood cultivars are increasingly used as landscape plants because they lack the disease and insect problems typically associated with the native flowering dogwood (Trigiano, personal comm.).

3.14 Cornus kousa Insect and Disease Problems

Kousa dogwood cultivars are increasingly used as landscape plants because they lack the disease and insect problems typically associated with the native flowering dogwood (Trigiano, personal comm.). Unlike *C. florida*, C*. kousa* is usually not very susceptible to powdery mildew and dogwood anthracnose. The native flowering dogwood, *C. florida*, is much more susceptible to anthracnose than is *C. kousa*, or hybrids of *C. florida* and *C. kousa. Cornus kousa* is resistant, but not immune to anthracnose and caution should be taken when making blanket claims of resistance (Witte et al., 2000) . A few seedling lines of *C. kousa* were found to be very susceptible (Windham and Trigiano, 1993). Kousa dogwoods have been recommended for planting in sites where flowering dogwoods have died from anthracnose caused by *Discula destructiva* Redlin (Gilman and Watson, 1993; Pecknold et al., 2001). Although, Kousa

dogwood leaves may be infected by this pathogen, little or no permanent damage is sustained (Santamour et al., 1989). Additionally, most *C. kousa* and *C. kousa x C. florida* crosses appear to be resistant to powdery mildew caused by *Erysiphe* (*Microsphaera) pulchra* (Klein et al., 1998).

Dogwood canker (etiology unknown) has never been observed on kousa cultivars (Trigiano, personal comm.; Windham, personal comm.) however, the plant has been reported to be susceptible to this disease. Gilman and Watson (1993) reported that early symptoms present smaller and paler leaves and infected branches turn red earlier in the fall. The foliar symptoms appear initially on the side of the canker and gradually spread as the canker becomes enlarged.

Dogwood crown canker, caused by *Phytophthora cactorum* (Lebert & Cohn) J. Schröt., is usually associated with wet soil conditions and all dogwoods (as well as most hardwoods) are susceptible. A generally poor appearance of the tree may be the first symptoms of infection by *Phytophthora.* Later, a canker appears that may contain reddish-brown ooze. The bark eventually sloughs off revealing wood with blue-black or reddish-brown streaks below. Twigs and branches on the side of the tree above the canker die first, but the entire tree will succumb when the canker has girdled the trunk (Douglas and Cowles, 2004). Phytophthora damage is rarely seen on Kousa dogwood and does not represent a major disease problem for this plant (Trigiano, personal comm.; Windham, personal comm.). Eliminating infected trees from areas with other susceptible dogwood trees or their replacement with resistant cultivars can reduce possible infestation of this fungus.

Leaf and flower blight (*Botrytis cinerea* Pers.: Fr. P) is a common disease of older kousa bracts. This fungal disease can be a serious problem during wet spring weather and the symptoms can range from a spotting of the flower bracts to a complete collapse of the bracts (Malinoski and Clement, 2003). Over time, infected bracts become covered with a gray fuzzy growth of fungus, which gives this fungal organism the common name 'gray mold'. Infected bracts that fall onto leaves can also cause leaf and twig infections (Malinoski and Clement, 2003) .

Under severe heat and draught conditions, Kousa foliage can scorch (Auge et al., 2002) creating a serious problem in the southeastern United States (Figure 3.3). Nevertheless, Kousa dogwoods seem to be more draught resistant than flowering dogwood (Dirr, 1998).

Dogwood borer (*Synanthedon scitula* Harris) damage was reported (Dirr, 1998), however no serious disease or insect damage was described and Kousa dogwood may have some resistance to this pest (Douglas and Cowles, 2004).

3.15 Cornus kousa Propagation Methods

Generally, *C. kousa* cultivars are propagated in a similar fashion to *C. florida,* including propagation from greenwood cuttings in spring, hardwood cuttings in summer, seed, layering and micropropagation. Usually, seeds have various dormancy conditions and some require either fall planting or a stratification period of approximately four months at 4°C (Hartmann et al., 2002). Germination, especially of stored seed, can be very slow. However, if the seeds

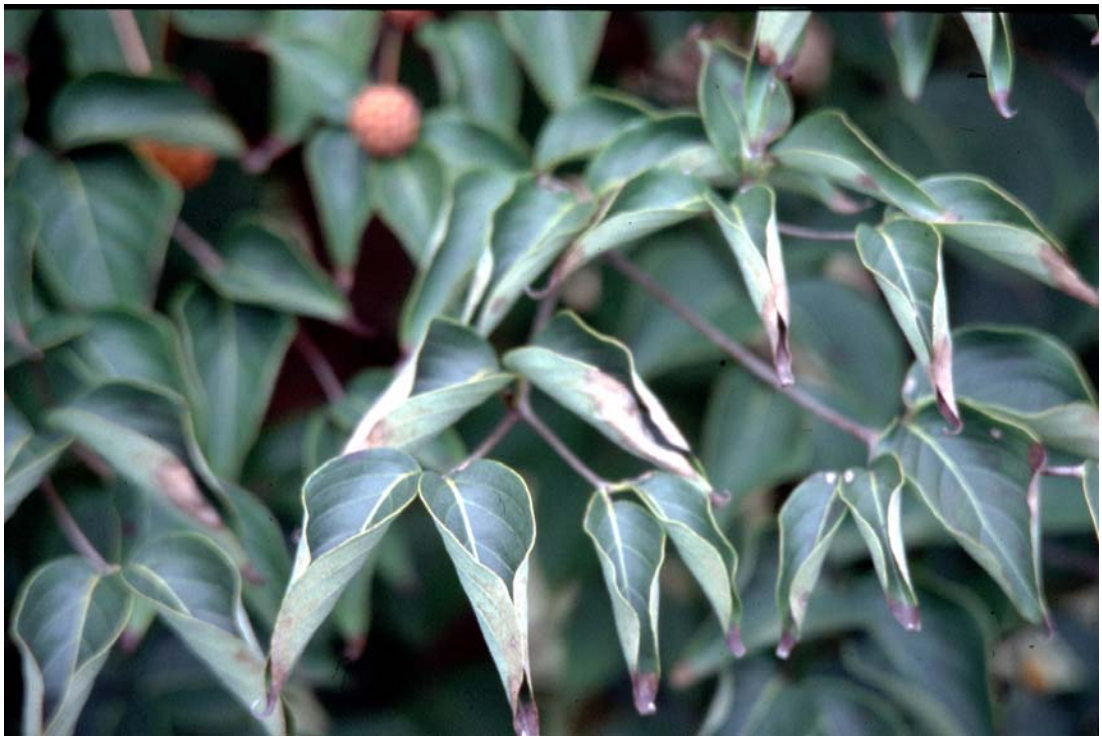


Figure 3.3. *Cornus kousa* leaf scorch and curl (courtesy of Mark Windham, University of Tennessee).

are gathered as soon as the fruit starts to develop color, the chances for better germination are often met. Seedlings can vary greatly in character, with some being far more vigorous than others (Fordham, 1990). Also, the seeds must be separated from the fruit pulp because it presumably contains germination inhibitors (McMillan-Browse, 1985).

Cornus kousa can also be propagated by cuttings, although these are not as vigorous as seedlings (Hartmann et al., 2002). Fordham (1990) concluded softwood cuttings root readily and therefore vegetative propagation of selected clones by cuttings is far more satisfactory. Cuttings collected in mid-June through July for northern states or from May through August in the southern parts of the United States, root well when wounded on one side and treated with rooting phytohormones (Hartmann et al., 2002). To ensure maximum survival of the cuttings during the winter period in the colder climates, the potted cuttings should be maintained in a heated cold frames or polyhouses with a temperature ranging between 0 to7°C (Hartmann et al., 2002).

Fordham (1984) also indicated that grafting can be another viable method of propagation, and *C. kousa* and *C. florida* can be reciprocally grafted. However, some graft incompatibility has been noted between some cultivars of *C. florida* and kousa rootstock and vice versa (Trigiano, personal comm.).

3.16 Cornus kousa Tissue Culture

Clonal selection followed by vegetative propagation is a very important strategy for plant improvement. The best plant specimen or simply, the finest individual seedlings can be selected from within an entire population for a specific purpose and reproduced by vegetative propagation. This will promote clonal multiplication into a population of the same identical genotype without limit. Most cultivars selected as clones are genetically heterozygous, so their uniqueness would be lost in the next seed propagated generation (Hartmann et al., 2002).

One of the reasons certain *Cornus sp*. cultivars were selected for their elite, unique and above all, superior performance in trials, disease resistance and drought tolerance, all of which are highly important for the green industry. As a result, faster and more efficient methods of propagation could be accomplished by using tissue culture techniques and all of its advantages in dogwood commercial propagation. In this research project, the following five *C. kousa* cultivars were included in the study*:*

a. ‘Rosabella’, has fine, rose pink bracts. This cultivar was offered though Wayside Gardens, and according to Dirr (1998) is the same as ‘Satomi’ (‘Miss Satomi’).
b. ‘Samaritan’ is one of the newest introductions and has white bracts, vigorous growth, radiant pink and burgundy fall color and is an offspring of

'National'. Variegated creamy white and green foliage remains attractive all summer (Witte et al., 2000).

c. ‘Heart Throb’ is a large red-bracted form with each inflorescence almost four inches wide. The cultivar was introduced by Don Schmidt Nursery, Boring, OR. A very new introduction ('Schmred') with a deep red, burgundy bloom, but flower color is not as intense in the mid-South as in the Far West (Witte et al., 2000).

d. ‘Little Beauty’ has a dense habit with red/purple flower color in the fall and was introduced by North Carolina State University Arboretum (Dirr, 1998). The tree is a shrubby form collected from wild Korean seed, distributed by North Carolina State University Arboretum (Witte et al., 2000).

e. ‘Christian Prince’

Recently, ‘Heart Throb’, ‘Rosabella’ and ‘Miss Satomi were demonstrated to have significant cultivar synomomy using DNA amplification fingerprinting (DAF) (Caetano-Anolles and Bassam, 1991), These cultivars were nearly identical and either were released under different names or very closely related full siblings (Trigiano et al., 2004).

3.2 Materials and Methods

3.21 Cornus kousa Explant Preparation

Cornus kousa stock plants that were used in this study were 2-3 years old. They were planted in 10-gallon pots and placed in the nursery area located at the University of Tennessee campus in Knoxville, TN. Plants were hand watered, avoiding disease problems associated with overhead irrigation. In January of 2003, plants were moved to the greenhouse and in the spring (March), before flowering, explants from young Kousa trees were collected. *Cornus kousa* cultures were established from axillary and apical buds from the following five cultivars: 'Little Beauty', 'Samaritan', 'Heart Throb', 'Rosabella' and 'Christian Prince'. Nodes were surface disinfected by soaking in 20% v/v commercial bleach solution (Clorox) amended with 0.1%Triton X-100 with constant stirring for eight to ten minutes and finally rinsed three times with sterile distilled water. After surface disinfestations procedures, 2mm of the cut ends that were exposed to the bleach were removed, and the explants were placed in polystyrene, disposable 60x20 mm petri dishes, containing either of two different media: ½ broad leaved tree medium/woody plant medium (BW) (Sato, 1991) (Appendix 5) or woody plant medium (WPM) (Lloyd and McCown, 1980) (Appendix 2). Multiplication media was amended with 8g/L of agar and the following concentrations of 6 – benzylaminopurine (BA): 0, 2, 4, and 8 µM. The pH of the medium was adjusted to 5.8 prior to autoclaving. Explants were then transferred

at four-week intervals for 28 weeks beginning in April onto fresh medium. Cultures (Figure 3.4.) were maintained in incubators at 23°C with 55 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ light intensity for 16/8 light-dark photoperiod. Basal callus was excised after each transfer to fresh medium, and terminal shoot tips were removed from explants to reduce apical dominance and stimulate axillary shoot growth from lateral buds. After the third cycle, all explants were placed on medium containing 2 µM BA, since it was previously reported by Kaveriappa et al. (1997) that shoot production from *C. florida* had been significantly enhanced at concentrations

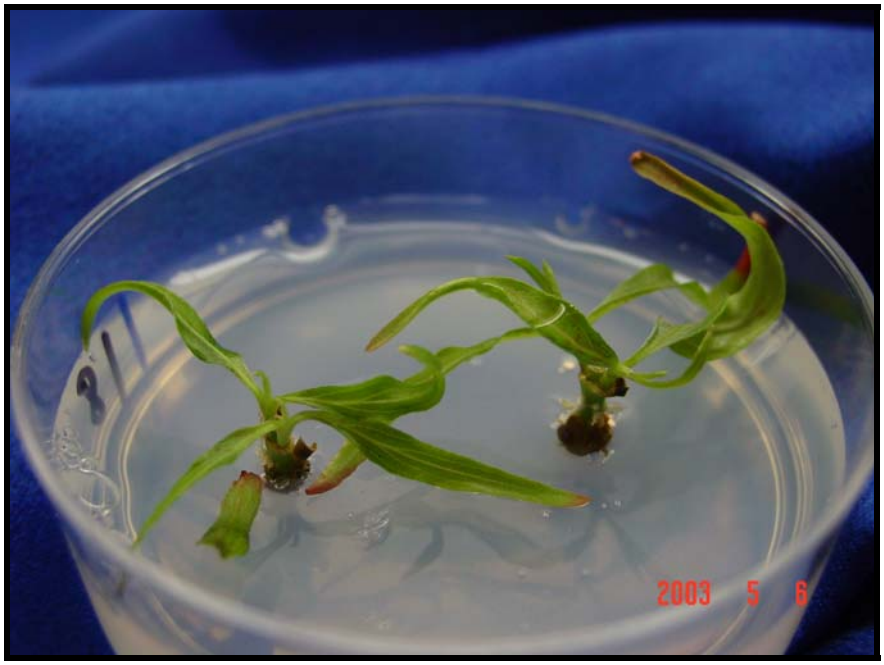


Figure 3.4. A culture of *Cornus kousa* 'Rosabella' apical and axillary buds after one cycle on ½ broad leaved tree medium/woody plant medium (BW) medium supplemented with 8 µM of BA in a 60 x 20 mm petri dish.

between 2.2 and 4.4 µM BA. Explants were acclimatized to *in vitro* conditions for six to seven culture cycles before the microshoots were used for rooting experiments.

3.22 Experimental Procedure

Microshoots, at least one centimeter long, were excised from proliferating cultures maintained on both WPM and BW media supplemented with 2 µM BA. Four week old microshoots from 'Samaritan', 'HeartThrob', and 'Rosabella' cultures were harvested and placed on either WPM or BW basal media supplemented with 7g/L of agar in 17x100 mm plastic, disposable culture tubes with plastic snap-on caps (Fisher Scientific, Atlanta Georgia). Each tube contained about 5 ml of one of the experimental root inducing media. Basal media were amended with either 1- naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), or indole-3-acetic acid (IAA) at the following concentrations: 0, 0.5, 1.5, 4.5 and 13.5 µM. Plant growth regulators were filter-sterilized using a 0.22 µm syringe filter (Fisher Scientific, Atlanta Georgia) and added after the media had cooled, but not hardened. This was done in order to keep the experiment uniform for all treatments. 'Little Beauty' and 'Christian Prince' did not perform well in the multiplication phase of tissue culture and were excluded from further studies. The cultures were placed in racks in a growth room at 21°C for six weeks with 55 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ light intensity for 16/8 light-dark photoperiod.

Proliferating masses were placed on fresh medium with 2 µM BA and were used again for the rooting projects.

3.23 Experimental Design

A preliminary rooting experiment employed a factorial arrangement of three growth regulators (IAA, NAA and IBA), five concentrations (0. 0.5, 1.5, 4.5 and 13.5 µM) of each growth regulator and two basal media (WPM and BW) (3x5x2) in a randomized complete block design (RCBD). The experimental unit consisted of one microshoot per culture tube. In RCBD, treatments are grouped into blocks that contain at least one replicate from each treatment. Experimental units are randomized within blocks, each employing a separate randomization scheme. This design was selected because it minimizes variability within a block while maximizing variability among blocks (Compton, 2000). After six weeks, data were collected as to how many microshoots rooted on the various treatments. Only rooted explants were observed and recorded.

3.24 Acclimatization of Microshoots

After six weeks on rooting medium (Figure 3.5.), whole plants were washed with tap water to remove agar and placed in Jiffy peat pellets (Jiffy products (N.B) Ltd. Shippagan, Canada), which were hydrated with warm tap

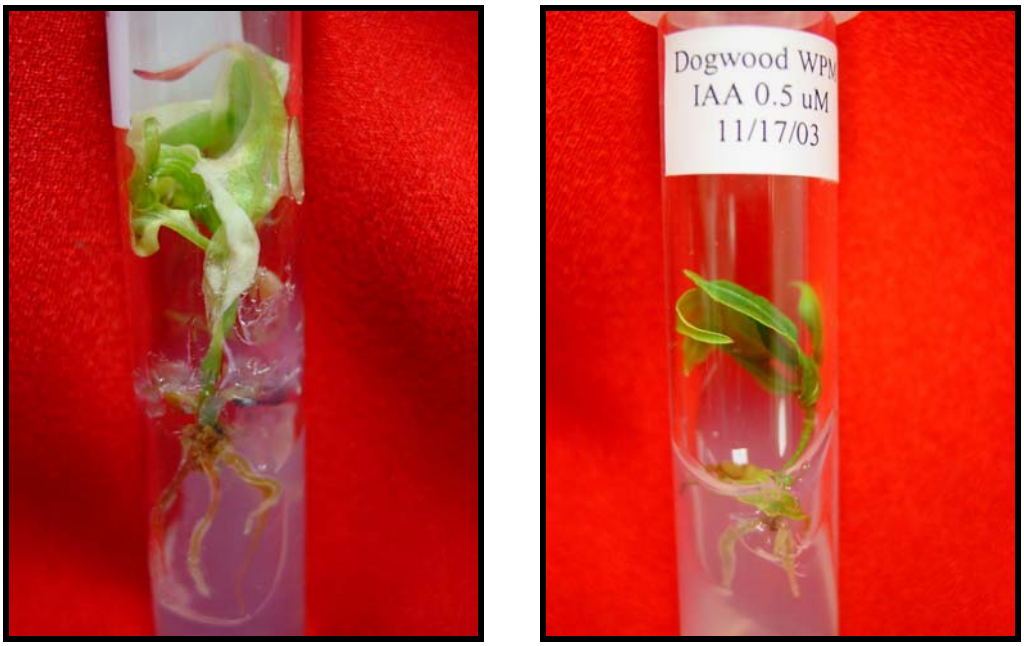


Figure 3.5. Cornus *kousa* ‘Samaritan’ and ‘Rosabella’ microshoots after six weeks on rooting medium, from left to right, placed on BW and WPM, amended with 13.5 and 0.5 M IAA respectively. Note the adventitious roots on both plantlets.

water until they were expanded and soft. Rooted microshoots were gently placed in the peat pallets to avoid breaking and damaging of the roots. Peat pellets with entire plants were then placed in Magenta GA-7 vessels for acclimatization and incubated in racks in the growth room at 21°C with 55 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ light intensity for 16/8 light-dark photoperiod.

Regenerated plantlets were initially watered with tab water only and thereafter when needed with 10% WPM basal salts without sucrose. The lids of the boxes were cracked open over a four-week period gradually adapting plants

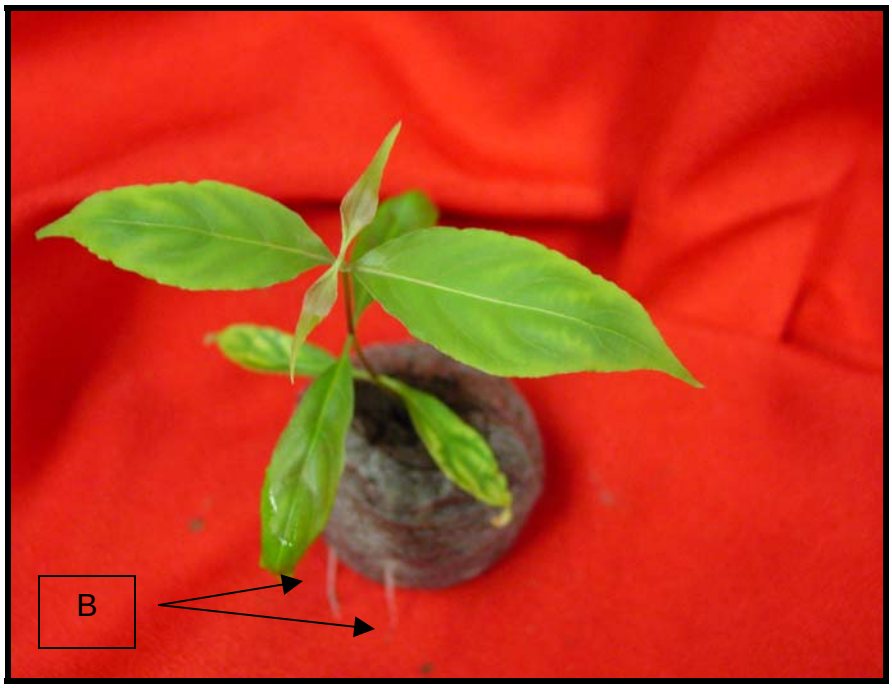
to a non-sterile, ambient environment. Washed plants easily were transferred to peat pellets (Figure 3.6.) and within four weeks were acclimatized to ambient conditions of the growth room. After total of 12 weeks, acclimatized plants were placed in 15 cm in diameter round pots containing pine bark medium (Appendix 6) (Windham and Witte, 1998) obtained from the University of Tennessee greenhouse and nursery production area. Plants were maintained under growth room environment for additional eight weeks after which they were planted in the landscape.

3.25 Results and Discussion

Several *Cornus* species have been propagated via tissue culture including *C. florida* L. (Flowering dogwood) (Kaveriappa et al., 1997; Sharma et al., 2005), *C. nuttalli* Audubon ex Torr. & Gray (Pacific dogwood) (Edson et al., 1994), and *C. canadensis* L. (Bunchberry dogwood) (Pennell, 1983). Kaveriappa et al. (1997) reported the first successful micropropagation protocol for *C. florida* from seedlings.

Many axillary propagation schemes employ BA concentrations between 1 and 10 µM to induce proliferation of new axillary shoots (Edson et al., 1997). However, concentrations above 4.4 µM of BA may inhibit proliferation and growth of axillary shoots. Furthermore, prolonged culture on induction medium resulted in drying of the tip and extensive callusing at the basal end of the elongated shoots (Rout and Das, 1993).

Figure 3.6. *Cornus kousa* cultivars placed in Jiffy peat pellets. A. 'Samaritan' microshoot was rooted on Woody Plant Medium (WPM) supplemented with 4.5 µM of IAA. Plant shown is before acclimatization to the growth room environment. B. 'Rosabella' microshoot was rooted on ½ broad leaved tree medium/woody plant medium (BW) supplemented with 0.5 µM IAA and acclimatized to ambient growth room conditions after four weeks. Note that the root system has grown beyond the peat pellet; C. 'Rosabella' was rooted on WPM supplemented with 0.5 µM IBA and acclimatized to ambient growth room conditions after four weeks.



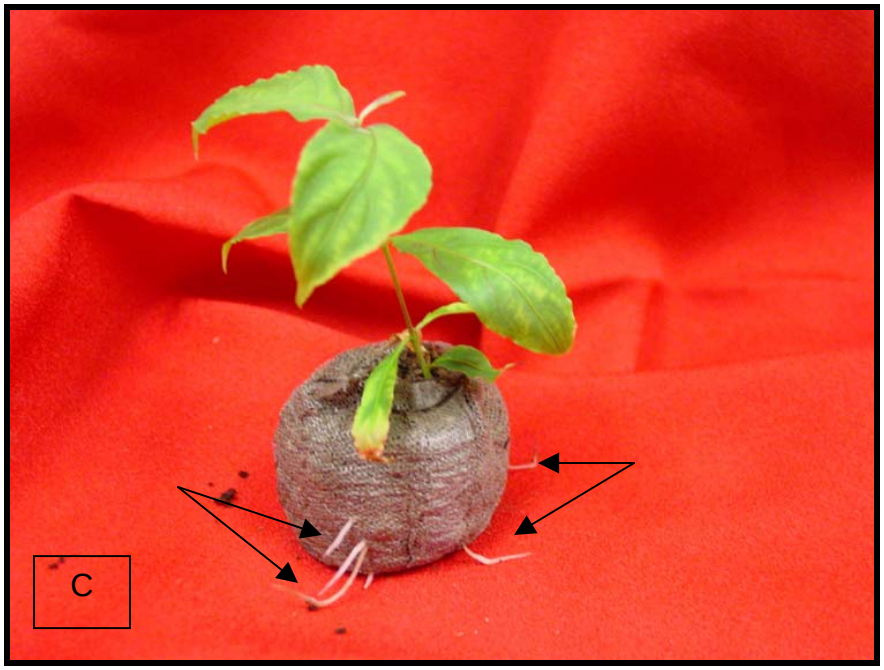


Figure 3.6. Continued.

Elongated shoots of *C. kousa* cultivars were placed on 2 µM BA after the third cycle, since it was previously reported by Kaveriappa et al. (1997) that shoot production and shoot quality for *C. florida* were significantly enhanced at BA concentrations between 2.2 and 4.4 µM. Similar BA concentrations were used in microshoot proliferation of *C. florida* (Declerck and Korban, 1994; Sharma et al., 2005; Trigiano et al., 1992a) and *C. nuttalli* (Edson et al., 1994). Lower concentrations of growth regulations have proven beneficial for shoot multiplication in a number of tissue culture systems for woody plants. For example, 2.2 µM BA in MS medium used in a micropropagation protocol for *Nonthapodytes foetida*, a small medicinal tree found in the rain forest of South India and Sri Lanka, improved shoot elongation (Ravisiiankar Rai, 2002). Other studies reported similar findings for maximizing shoot proliferation (Jain and Babbar, 2000; Ravisiiankar Rai, 2002; Rout and Das, 1993).

During the proliferation phase of *C. kousa* cultivars, microshoots were placed in petri dishes in pairs since in previous experiments and preliminary trials better shoot induction for *C. florida* was achieved (Trigiano, personal comm.). Similar practices were employed in a study of bamboo (*Dendrocalamus hamiltonii* Nees et Arn. Ex Munro) explants where microshoots placed in a unit of three to four, responded better than the individual explant alone (Sood et al., 2002).

The ability of shoots to form roots is influenced by several factors, including genotype (Haissig, 1986), the level of tissue maturity (Bonga, 1982; Sharma et al., 2005; Trigiano et al., 1992a), as well as physiological

characteristics due to seasonal constrains and changes (Howard, 1996; Marks and Simpson, 2000). Adventitious root development on microshoots is dependent on the composition of the basal medium and auxin concentrations. Three types of roots are typically formed in culture -- unbranched profuse rooting with sparse roots, elongated less hairy white roots with secondary and tertiary roots, and thick, slightly brownish finger like roots (Sood et al., 2002). This study concentrated on determining basal medium composition and auxin (type and concentration) that would induce adventitious roots on C. kousa microshoots.

After six weeks, data were collected from the rooting project where treatments consisted of a factorial arrangement of three growth regulators (IAA, NAA and IBA), five concentrations (0, 0.5, 1.5, 4.5 and 13.5 µM) of each growth regulator and two basal media (WPM and BW). Only rooted explants were analyzed and recorded, and the number of roots per rooted microshoot was counted. Although several repetitions of the experiment were conducted, statistical analysis was not completed due to insufficient data. However, initial results did reveal some trends.

The results indicated NAA and IAA appeared to be better for adventitious root production by *C. kousa* cultivars microshoots than IBA. Contrary to this finding, *C. florida* microshoots generated the most adventitious roots when exposed to continuous lower concentrations of either 2.5 or 4.9 µM of IBA over a four week period (Kaveriappa et al., 1997; Sharma et al., 2005). Preliminary results showed a total of nine microshoots rooted on both WPM and BW media supplemented with IBA, 17 microshoots rooted on media supplemented with

NAA, and 14 microshoots rooted on media supplemented with IAA (Table 2). However, the majority of roots formed on *C. kousa* microshoots were similar to the third category described by Sood et al., (2002) - thick, slightly brownish finger like roots. Rout and Das (1993) also reported that among the three auxins tested (IAA, NAA, and IBA), only IBA at the concentration of 4.9 µM induced rooting, resulting in three to four roots per rooted microshoot. A mean of four to five adventitious roots formed on 46% of the *C. florida* microshoots using 2.5 or 4.9 µM IBA (Kaveriappa et al., 1997). A comparatively lower average number of roots formed on *C. kousa* microshoots exposed to various concentrations of IBA (Table 2). 'Samaritan' microshoots produced an average of about 6.2 roots on both WPM and BW media supplemented with various concentrations of NAA. 'Samaritan' microshoots exposed to NAA formed the highest number of formed roots among all tested treatments for the three cultivars. Among the three auxins used to induce roots on 'Heart Throb' microshoots, NAA resulted in the best yield. For this cultivar, an average of 2.3 roots per each microshoot were formed when exposed to different NAA concentrations and resulted in the highest average number among all treatments tested for this particular cultivar. Interestingly, 'Rosabella', which is very similar to 'Heart Throb' genetically (Trigiano et al., 2004), microshoots did not produce any roots on BW media supplemented with NAA. However, 'Rosabella' produced an average of 3.3 roots per rooted microshoot when exposed to different IBA concentrations. All three cultivars, when exposed to various IAA concentrations resulted in the lowest

Table 2. Effects of IBA, NAA and IAA on root formation from *in vitro Cornus kousa* microshoots.

C. kousa cultivars	Medium	IBA (μM)					NAA (μM)					IAA (μM)				
		0	0.5	1.5	4.5	13.5	0	0.5	1.5	4.5	13.5	0	0.5	1.5	4.5	13.5
Samaritan	WPM	0/3	1/3	1/3	0/3	0/3	0/3	2/3	2/3	3/3	2/3	0/3	2/3	0/3	1/3	0/3
Samaritan	BW	0/3	0/3	1/3	0/3	1/3	0/3	0/3	0/3	2/3	0/3	0/3	0/3	0/3	1/3	2/3
Heart Throb	WPM	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	1/3	1/3	0/3	0/3	0/3	0/3	0/3
Heart Throb	BW	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3	1/3
Rosabella	WPM	0/3	1/3	1/3	0/3	0/3	0/3	0/3	1/3	0/3	1/3	0/3	1/3	0/3	1/3	0/3
Rosabella	BW	0/3	0/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	1/3	2/3	1/3
		0	3	5	0	1	0	2	4	7	4	0	4	1	5	4
Total		9					17					14				

average number of roots formed on rooted microshoot (1.8, 1.5 and 1.45 for ‘Samaritan’, ‘Heart Throb’ and ‘Rosabella’, respectively) (Table 3). Further research is necessary to confirm which auxins are best suited for rooting of microshoots of each kousa cultivar. This study can be used as a guideline for further trials related to different *C. kousa* cultivars.

Hackett (1985) states the success of either shoot or root formation *in vitro* is often related to the maturity of the donor plant. Sharma et al. (2005) confirmed the finding that age of microshoot had a significant effect on rooting of *C. florida* explants. They concluded that five, six and seven week old microshoots had high and statistically similar rooting percentage of 83% (Sharma et al., 2005). Although, the same procedure was used in earlier Kaveriappa et al. study, (1997) only 46% of the four week old microshoots formed roots. Sharma et al. (2005) stated that possible reason for the are related ability of these microshoots to form roots could be a change in the auxin:cytokinin ratio at the site of root initiation. After four to five weeks, cytokinins from the subculturing media are probably depleted and lost from stem tissues as well. This loss of cytokinin presumably could be a reason for increased rooting efficiencies of *C. florida* microshoots.

Similar to earlier reports with *Cornus* species (Edson et al., 1994; Kaveriappa et al., 1997; Sharma et al., 2005), the basal medium influenced subsequent rates of microshoot proliferation. In this study, both WPM and BW media supported rooting of microshoots however, WPM appears to support more root production, compared to BW, which is similar to *C. florida* Kaveriappa et al.,

Table 3. Effect of IAA, NAA, and IBA on average number of adventitious roots formed per each rooted microshoot.

C. kousa cultivar	**Auxin used**	**WPM**	**BW**	Average
Samaritan	IAA	1.6	2	1.8
Samaritan	NAA	3.45	9	6.23
Samaritan	IBA	3	3	3
Heart Throb	IAA	1	2	1.5
Heart Throb	NAA	2.67	2	2.33
Heart Throb	IBA	0	2	2
Rosabella	IAA	1.5	1.4	1.45
Rosabella	NAA	2	0	2
Rosabella	IBA	4.5	2	3.25

(1997) study. Kaveriappa et al., (1997) concluded WPM was superior to either MS or SH (Schenk and Hildebrandt, 1972) for root formation in *C. florida*.

A greater number of ’Samaritan’ and ‘Heart Throb’ microshoots rooted on WPM amended with a wide range of NAA concentrations, whereas more ‘Rosabella’ microshoots rooted on BW medium amended with various concentrations of IAA (Table 2). Although insufficient data were gathered to separate media and auxin affects in this study, there is ample evidence in the literature to suspect that the basal medium affected rooting efficiency. For example, in a study with *N. foetida* (Ravisiiankar Rai, 2002), one-fourth strength MS amended with 2.4 µM IBA combined with 5.7 µM IAA was the most effective treatment for inducing adventitious roots compare to other treatments employing different basal medium formulations and growth regulators. Since ‘Rosabella’ and ‘Heart Throb’ are very closely related (Trigiano et al., 2004), the two cultivars would be expected produce roots under similar conditions and exposure to similar treatments. However, additional trials and further research are necessary to confirm this finding.

Interestingly, microshoots placed on either basal media supplemented with NAA produced significant amount of callus compared to microshoots exposed to other growth regulator treatments.

Overall, rooted microshoots that were exposed to IBA treatments at lower concentrations of 0.5 and 1.5 µM resulted in best and most rooting (Figure 3.7.), whereas NAA and IAA treatments were inconclusive. ‘Samaritan’ preformed the best of all the tested cultivars (65% microshoots rooted on both media

Figure 3.7. Acclimatized, cloned and elongated *Cornus kousa* ‘Rosabella’ cultured *in vitro* on BW medium supplemented with 1.5 and 0.5 µM IBA (four and five months, respectively after rooting experiment was initiated).

Figure 3.7. Continued

supplemented with NAA, 45% rooted on IBA and 43% rooted on IAA).

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Appendices

Appendix 1. Composition of Murashige and Skoog (MS) medium in milligrams per liter and molar concentrations.

Macronutrients in mg/L (mM)

NH_4NO_3	1650 (20.6)
$CaCl_2 \cdot 2H_2O$	332.2 (2.3)
$MgSO_4 \cdot 7H_2O$	370 (1.5)
KNO_3	1900 (18.8)
KH_2PO_4	170 (1.3)

Micronutrients in mg/L (mM)

H_3BO_3	6.2 (100)
$CoCl_2 \cdot 6H_2O$	0.025 (0.1)
$CuSO_4 \cdot 5H_2O$	0.025 (0.1)
Na_2EDTA	37.3 (100)
$FeSO_4 \cdot 7H_2O$	27.8 (100)
$MnSO_4 \cdot H_2O$	16.9 (100)
KI	0.83 (5)
$Na_2MoO_4 \cdot 2H_20$	0.25 (1)
$ZnSO_4 \cdot 7H_2O$	8.6 (30)

Organics in mg/L (mM)

Myo-inositol	100 (550)
Glycine	2.0 (26.6)
Nicotinic Acid	0.5 (4.1)
Pyridoxine HCl	0.5 (2.4)
Thiamin HCl	1.0 (3.0)

* adapted from Trigiano and Gray, 1999 (Trigiano and Gray, 2000)

Appendix 2. Composition of Woody Plant Medium (WPM) medium in milligrams per liter and molar concentrations.

Macronutrients in mg/L (mM)

NH_4NO_3	400 (5.0)
$CaCl_2 \cdot 2H_2O$	96 (0.7)
$Ca(NO_3)_2 \cdot 4H_2O$	556 (2.4)
$MgSO_4 \cdot 7H_2O$	370 (1.5)
K_2SO_4	990
KH_2PO_4	170 (1.3)

Micronutrients in mg/L (mM)

H_3BO_3	6.2 (100)
$CuSO_4 \cdot 5H_2O$	0.25 (1)
Na_2EDTA	37.3 (100)
$FeSO_4 \cdot 7H_2O$	27.8 (100)
$MnSO_4 \cdot H_2O$	22.3 (132)
$Na_2MoO_4 \cdot 2H_20$	0.25 (1)
$ZnSO_4 \cdot 7H_2O$	8.6 (30)

Organics in mg/L (mM)

Myo-inositol	100 (550)
Glycine	2.0 (26.6)
Nicotinic Acid	0.5 (4.1)
Pyridoxine HCl	0.5 (2.4)
Thiamin HCl	1.0 (3.0)

* adapted from Trigiano and Gray (Trigiano and Gray, 2000)

Appendix 3. Composition of the general-purpose Pro-Mix 'BX' peat-based growing medium.

- Canadian Sphagnum Peat Moss (75-85 % / vol.)
- Perlite — horticultural grade
- Vermiculite
- Dolomitic & Calcitic Limestone (pH adjuster)
- Macronutrients
- Micronutrients
- Wetting Agent

* adapted from http://www.premierhort.com/

Appendix 4. Composition of Sunshine Professional Peat-Lite Mix #1 medium used for acclimatization of *Cladrastis kentukea*.

- Canadian Sphagnum peat moss
- Coarse grade perlite
- Gypsum (Ca SO_4)
- Dolomitic lime
- Wetting agent
- Miracle–Gro All purpose Plant Food 15-30-15 (1.5 tablespoons/ 1 gallon of water)

* adapted from http://www.sungro.com/

Appendix 5. Composition of modified ½ broad-leaved tree medium/woody plant medium (BW). (Adapted from Sato, 1991)

		BW	**Volume of BW**	**WPM**	**BW**	**0.5(WPM +BW)**
Stock	Salt	Stock (mg/L)	Stock (ml/L)	(mg/L)	(mg/L)	(mg/L)
Nitrate	NH_4NO_3	650	100	400	65	232.5
	KNO_3	1900			190	95
	$Ca(NO_3)_2$* $4H_2O$	6400		556	640	598
Sulfate	$(NH_4)_2SO_4$	2400	100		240	120
	$MgSO_4$*$7H_2O$	3700		370	370	370
	K_2SO_4	8600		990	860	925
	$FeSO_4$*$7H_2O$	278		27.8	27.8	27.8
	$MnSO_4$*$4H_2O$	223		22.3	22.3	22.3
	$ZnSO_4$*$7H_2O$	86		8.6	8.6	8.6
	$CuSO_4$*$5H_2O$	2.5		0.25	0.25	0.25
Halide	$CaCl_2$*$2H_2O$	440	100	96	44	70
	KI	1.5			0.15	0.075
	$CoCl_2$*$6H_2O$	0.2			0.02	0.01
EDTA	Na_2EDTA	186100	0.2	37.3	37.3	37.3
P, B, Mo	KH_2PO_4	1700	100	170	170	170
	H_3BO_3	62		6.2	6.2	6.2
	Na_2MoO_4* $2H_2O$	2.5		0.25	0.25	0.25
Vit.	Pyridoxine HCl	500	1	0.5	0.5	0.5
	Thiamine HCl	1000		1	1	1
	Nicotinic Acid	500		0.5	0.5	0.5
	L-glycine	2000		2	2	2
Myo Glut	Myo-inositol	20000	5	100	100	100
	L-glutamine	400			2	1

Appendix 6. Composition of pine bark medium used for acclimatization of *Cornus kousa* cultivars.

- 1.45 kg/m^3 dolomitic limestone
- 0.83kg/m^3 0-46-0 super phosphate
- 0.94 kg/m^3 gypsum (Ca SO_4)
- 0.62 kg/m^3 Micromax (Grace Sierra, Milipitas, CA)
- 0.83 kg3m^3 epsom salts ($MgSO_4$)
- Fertilizer: 0.62 kg/m^3 18-6-12

* adapted from Windham and Witte (Windham and Witte, 1998)

Vita

Denita Hadziabdic was born in Sarajevo, Bosnia and Herzegovina, on November 10th 1978. In Sarajevo, she finished both elementary and high school and in 1997 she began studying at the College of Forestry, Department of Horticulture at the University of Sarajevo. As a sophomore, she received numerous scholarships and transferred to Tennessee Technological University. She received her Bachelor's degree in Agriculture-Horticulture from the School of Agriculture at Tennessee Technological University in December 2002.

In January of 2003 she started her graduate studies and worked as a graduate research assistant at the University of Tennessee, Department of Plant Pathology and Entomology under Dr. Robert N. Trigiano. She received her Master's degree in May 2005.

Denita is a member of the Mortar Board, National Honor Society, Delta Tau Alpha, National Agricultural Honor Society, Gamma Sigma Delta, National Agricultural Honor Society, Phi Kappa Phi, National Honor Society and Omicron Delta Kappa, National Leadership Honor Society.

She also received several recognitions including the Scotts Company National Scholar, American Society for Horticulture Science Scholars Award, and the Tennessee Agricultural Production Association Outstanding Undergraduate and Graduate Student Awards.

Denita will start working as a plant disease diagnostician at Louisiana State University in Baton Rouge, LA upon her graduation.