This is to certify that the

dissertation entitled

Tissue Culture and Agrobacterium - mediated Transformation of Gleditsia triacanthos L. (honeylocust)

presented by

Lilian B. Ungson

has been accepted towards fulfillment
of the requirements for

Doctoral degree in Forestry

Major professor

Date April 5, 1995

TISSUE CULTURE AND *Agrobacterium*-MEDIATED TRANSFORMATION OF *Gleditsia triacanthos* L. (HONEYLOCUST)

By

Lilian B. Ungson

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Forestry

1995

ABSTRACT

TISSUE CULTURE AND *Agrobacterium*-MEDIATED
TRANSFORMATION OF *Gleditsia triacanthos* L. (HONEYLOCUST)

By

Lilian B. Ungson

Micropropagated plantlets from mature trees and gene transfer to this species have not been obtained. These experiments focused on these problems. The objectives of the research were to devise methods to micropropagate honeylocust from explants of juvenile and mature plants and to examine the feasibility of gene transfer to honeylocust via *A. tumefaciens* and *A. rhizogenes* transformation systems.

Explant types evaluated included cotyledon and hypocotyl from sterile germinating seedlings, nodal explants from 8 and 10 month-old seedlings, dormant buds from crown shoots, nodal explants from root suckers, stump sprouts and elongating crown shoots. Callus formed at the cut edges of cotyledon and hypocotyl explants grown in MS media supplemented with various levels of BAP (5,10,15 and 30 μM). Shoot buds formed in cotyledon and hypocotyl explants. Rooting occurred using cotyledon explants. Shoot elongation was promoted in lower (1 μM) BAP level. MS medium with 1 μM BAP promoted bud emergence and elongation of nodal explants in 8 and 10 month-old seedlings. Buds from nodes nearer the root were the least responsive.

Dormant buds cultured with bud scales intact or excised failed to show elongation at various levels of BAP. Buds in root sucker segments and stump sprouts elongated in MS media containing reduced nitrates and supplemented with BAP, zeatin, NAA

and GA. A lower percentage of elongation was obtained in nodal explants from summer crown shoots.

Rooting of micropropagated shoots (rom cotyledonary nodes and hypocotyl segments, nodal segments of seedlings, root suckers and stump sprouts) was obtained in MS medium containing nitrates at half strength, supplemented with IBA or NAA or both auxins. Cultures were incubated in a 9-day dark period and subcultured in an auxin-free modified MS media in an alternating light/dark regime.

Tumors and hairy roots were observed in tissues inoculated with *A. tumefaciens* and *A. rhizogenes*, respectively. Continued growth and proliferation of the tumors occurred in medium lacking exogenous phytohormones and octopine was detected in *A. tumefaciens*-induced tumors. It was recommended that future work should include not only the two restriction enzymes used but more restriction enzymes to help find conclusive evidence of T-DNA integration into the honeylocust genome.

DEDICATION

This dissertation is dedicated to my parents- Antonio and Eugenia Ungson; brother- Antonio; sisters- Olivia, Yvonne and Susan, and my friend- Teresita de Guzman.

ACKNOWLEDGMENTS

Several persons helped me pursue, persevere in, and finish my graduate program. I am very thankful and grateful to all of them: Dr. Daniel Keathley, my major professor for guidance and support during the course of my studies and to the other members of my committee--Dr. Michael Gold, Dr. James Kielbaso and Dr. Kenneth Sink for their valuable suggestions and advice on my draft dissertation; Dr. Carl Ramm for advice on statistics; Dr. Nyuyen Phu for solutions to some problems and the late Dr. James Hanover for his constant encouragement during my course work.

Andrew David answered many questions and generously gave his time, knowledge and experience in various facets of my research and thesis preparation.

Several colleagues and friends shared their time and abilities in various endeavors - they helped make things "doable": Reynaldo Ebora, Margaret Payne, William Linnell, Chishi Chu, Maria Ioannidou, Mark Hare, Abigail Eaton, Jose Pinto, John Davis, Kyung-Hwan Han, Roy Prentice, Randy Klevickas, Paul Bloese, Joseph Zeleznik, Putera Parthama, Djoyo, Yahyu, Anne Hughes, Joyce Schubel, Barbara Anderson, Jean Ecker, Karin Miller, Claire Elouard, Dr. and Mrs. Fernando Caburnay and Yiyi Chen.

Filipino friends at Michigan State University shared their pleasant experiences in graduate school and helped make my life in the U.S. productive and enjoyable: Reynaldo and Madeline Ebora, Glenda Soriano, Sue Liza Saguiguit, Jo-Anne Palma,

Rodolfo and Leticia Altamirano, Cheribeth Tan, Emmanuel Viray, Rex and Vangie Alocilja, Nick and Monina Uriarte, Ernesto and Isidra Guiang, Leah Cuyno, Cathy Obien, and Danilo and Annalie Campos.

I am also very thankful to the Michigan State University Grounds Maintenance, Physical Plant and Campus Park and Planning departments, and Cottage Nursery for providing information about honeylocust.

I acknowledge with thanks the financial support for my studies from WINROCK International Forestry/Fuelwood Research and Development (F/FRED) project and the additional funds provided by the Michigan State University Department of Forestry and the Office of International Students and Scholars. I am grateful to the Southeast Asian Ministers of Education Organization Regional Center for Tropical Biology (SEAMEO-BIOTROP) for endorsing my study at Michigan State University.

I appreciate the Dial-a-Ride service of the Michigan State University Automotive Services for bringing me home most nights.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

CHAPTER 2

Table Number

CHAPTER 3

Table Number

LIST OF FIGURES

INTRODUCTION

The adoption of agroforestry practices is one possible solution to many existing land-use problems involving energy, food and soil erosion. The components of agroforestry systems vary but by definition must include at least one woody perennial species (Huxley, 1983a). A major issue in developing agroforestry systems has revolved around the identification of the best trees to plant (Huxley, 1983b).

Smith (1950) recognized *Gleditsia triacanthos* (honeylocust) as one of the desirable trees for use in a system of "permanent agriculture" which in recent years has become popularly known as "agroforestry." Honeylocust belongs to the genus *Gleditsia*, a small genus of twelve species with representatives well distributed in temperate and subtropic North and South America (Allen and Allen, 1981). The natural distribution of this species is shown in Figure 1. Selected cultivars of *Gleditsia triacanthos* are widely planted for shade and ornamental use in the United States and Canada. Among those traits for which these cultivars were selected, are better crown form, thornless without pods, bright yellow spring foliage, golden yellow fall color and dark green summer hue. It is also cultivated as an ornamental in those areas where *Ulmus americana* has been seriously affected by the Dutch elm disease (Robertson and Lee, 1976). Its common name refers to the sweet, succulent pulp found in the seed pod (Funk, 1957).

Smith (1950) discussed the desirable qualities of honeylocust for use in agroforestry. It has strong, durable and beautiful wood principally useful for posts

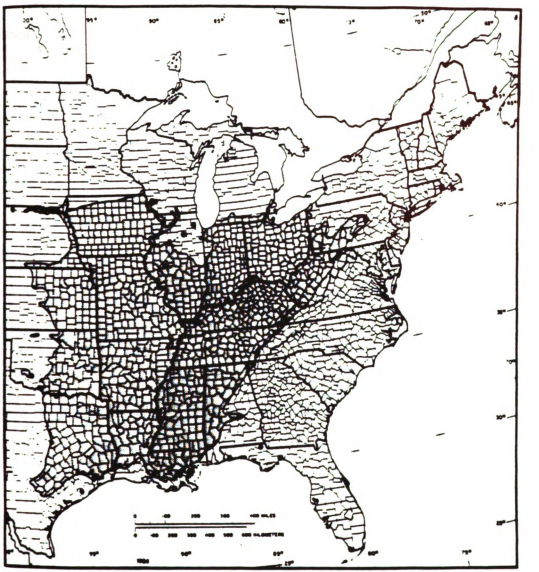

Figure 1. The native range of honeylocust (Blair, 1990) as indicated by the dark shaded areas.

and railroad ties. Under optimal site conditions, it grows rapidly giving an annual increase of two feet in height and one-half inch in diameter (Funk, 1957). It has an open crown through which much light can pass to plants underneath. Biennally, it bears a large seed crop which is easy to harvest. The seeds can even be ground and used as a dietary supplement for farm animals. The first cultivars selected in America were for the potential use of their pods as cattle food (Santamour, 1978). Bagley (1976) recognized the usefulness of honeylocust as a multipurpose crop tree for shelterbelts in the Great Plains. Williams (1982) mentioned this species as a staple perennial crop tree for marginal lands in the Appalachians as it can provide a source of fodder protein, energy and can be effectively used for erosion control. Honeylocust is also an excellent tree for pasture planting, since the shade cast by its feathery foliage is heavy enough for livestock but allows sufficient light to pass through to promote good grass growth. Selection of new clones of high protein content is being undertaken for winter grazing by sheep (Dupraz and Baldy, 1994). Two clones which were discovered in 1934 - Millwood (originated in North Carolina) and Calhoun (originated in Alabama) yielded 31% and 36% pod sugar (dry weight), respectively (Chase, 1947).

Gold (1984) discussed management scenarios for its use: (1) as a component in multipurpose shelterbelt system; (2) as a perennial crop tree for marginal lands; (3) for use in watershed management and for erosion control; (4) in two-tier, multi-cropping systems, and (5) in short-rotation intensive-culture systems.

Ease of propagation is an important consideration in identifying suitable tree species for agroforestry (Huxley, 1983a). Honeylocust is easily regenerated from

seed. Honeylocust seeds remain viable for several years if stored dry at 1 - 4°C (Gold, 1984). For successful germination the seeds must be scarified to break dormancy prior to planting. This is usually accomplished by the use of concentrated sulfuric acid (Heit, 1967).

Honeylocust can also be propagated vegetatively. Efforts to find an inexpensive method of reproducing high quality clonal stocks for sources of food for livestock led to some observations on vegetative propagation of honeylocust (Stoutemeyer *et al.*, 1944). Root cuttings were found to be the most promising method of reproducing desirable strains in large quantities at reasonable cost. Root cuttings from mature trees however, sprout less vigorously than younger trees and should be avoided if possible (Stoutemeyer *et al.*, 1944). Nursery stock of some selections of honeylocust has been obtained largely by grafting and budding. In using these methods there is the risk of sprouting from the rootstock. The expenses incurred during production of grafted and budded stock are often so high that commercial producers can not sell at a price low enough to meet the needs of pasture plantings (Stoutemeyer *et al.*, 1944). Honeylocust stem cuttings have also been successfully used to propagate elite genotypes. Such cuttings root readily following application of indolebutyric acid, indoleacetic acid, or naphthalene acetic acid (Stoutemeyer *et al.*, 1944). Grafted or budded stock can be propagated by using hardwood or greenwood cuttings and then taking root cuttings from the trees after they have become thoroughly established (Stoutemeyer *et al.*, 1944). Although these methods of vegetative propagation are effective for honeylocust, current technology in tissue culture offers an alternative propagation means which could produce clones of selected genotypes in a shorter time.

Other potentials of clonal propagation through tissue culture include possibilities of early screening of growth rates and disease resistance and evaluation for tolerance of mineral-deficient soil or water-logged soil, adverse climatic factors or air pollutants (Burley, 1989; Srivastava, 1989).

Recent advances in biotechnology have made possible gene transfer for many forest species using a vector system such as *Agrobacterium tumefaciens* and *A. rhizogenes* (Fillatti *et al.*, 1987; Ahuja, 1988; Davis, 1989; Han, 1991). In this method foreign DNA is introduced into plant cells utilizing the natural gene-transfer system of the bacterium. This could be of value in honeylocust, particularly the gene (s) giving resistance to the serious mimosa webworm (*Homadaula anisocentra* Meyrick) attack where webworm larvae feed on the leaves (Bastian and Hart, 1990a and b). Two cultivars are reported to show some level of resistance to webworm damage (Dirr, 1977; Bastian and Hart, 1990a). These cultivars will be very important in future efforts to transfer resistance genes to non-resistant honeylocust trees possessing desirable characteristics for agroforestry.

Another important application of gene transfer in honeylocust would be the possibility of producing trees which would be compatible with *Rhizobium* symbiotic association. In simple terms, this would deal with genes involved in the nodulation process of the legume-bacterium symbiosis and the nitrogen-fixing (nif) genes. Thus, nitrogen-fixing activity of bacterial strains of this genus could be incorporated in honeylocust and bestow soil-improving properties. The ability to nodulate is absent within certain sections of the family Leguminosae, and the inability to nodulate is determined by basic phylogenetic factors of the host plant (Allen and Allen, 1981).

Absence of nodulation is a constant characteristic of most of the genera in *Caesalpinoideae* in which honeylocust is classified. Therefore, integrating nodulation genes from species of other phyletic lines in the same family to the honeylocust genome, while admittedly of extreme complexity, would seem to be more possible than from genomes of non-leguminous species.

One of the first steps in attempts for a possible genetic transformation using *Agrobacterium* is to determine whether honeylocust is a compatible host to *Agrobacterium* i.e., susceptible to infection. Infected plants and tissues are typified by manifesting the "crown gall" disease if infected by *A. tumefaciens* or "hairy root disease" if infected by *A. rhizogenes.*

It is also essential that an *in vitro* culture shoot regeneration system exist for production of transgenic plants. *In vitro* culture of honeylocust was studied by Rogozinska (1967, 1968, 1969). The studies showed that benzylaminopurine (BAP) alone or in combination with auxin initiated bud formation in cotyledons and that naphthalene acetic acid (NAA) had the strongest rhizogenic properties. Shoot and root differentiation was induced in the cotyledons. Rogozinska (1969) mentioned that the results pointed to a new way for growing and reproducing new varieties and forms of honeylocust. Some studies were conducted on seeds of the thornless variety of honeylocust (Sommer, personal communication). Shoot formation from callus was only sporadic, thus, an efficient protocol was not developed. In one instance cultures produced embryoids from callus induced on cotyledon explants cultured on MS medium containing BAP and NAA. However, the embryoids failed to germinate into mature plants.

In studies to date, regeneration of honeylocust plantlets from both juvenile and adult trees has not been attained. Successful gene transfer for this species has also not been reported. This dissertation focuses on these problems. The results of experiments on micropropagation using explants from germinating seeds, 8 and 10 month-old seedlings, juvenile and mature trees, and preliminary work in the development of a genetic transformation protocol are presented.

The overall objective of this research was to devise methods to micropropagate honeylocust from explants of juvenile and mature plants and to examine the feasibility of gene transfer to honeylocust via *Agrobacterium tumefaciens* and *A. rhizogenes* systems. The first chapter deals with regeneration of shoots of honeylocust from germinating seeds, seedlings, and juvenile and mature explants. The second chapter relates studies on the response of the regenerated shoots to root induction medium and transfer of rooted plantlets to soil. The third chapter presents investigations on infection of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* on honeylocust cotyledons and intact seedlings as well as the detection of possible integration of bacterial genes. The results indicate progress toward utilizing *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the introduction of genes for novel traits into honeylocust.

LITERATURE CITED

Ahuja, M.R. 1988. Gene transfer in forest trees. In: Genetic Manipulation of Woody Plants, Hanover, J.W. and D. E. Keathley, (eds.), Plenum Press, New York. pp. 25-41.

Allen, O.N. and E.K. Allen. 1981. The Leguminosae. The University of Wisconsin Press, Wisconsin, USA. pp. xiii-xxiii, 298-300.

Bagley, W.T. 1976. Multipurpose tree plantations. In: Shelterbelts on the Great Plains. Great Plains Ag. Council Publ. No. 78. Proc. of the Symp. Denver, April 20-22. pp. 125-128.

Bastian, R.A. and E.R. Hart. 1990a. Honeylocust clonal effects on developmental biology of mimosa webworm (Lepidoptera: Plutellidae). J. Econ. Entomol. 83:533-538.

Bastian, R.A. and E.R. Hart. 1990b. First-generation parasitism of the mimosa webworm (Lepidoptera: Plutellidae) by *Elasmus albizziae* (Hymenoptera: Eulophidae) in an urban forest. Environ. Entomol. 19:409-414.

Blair, R. 1990. *Gleditsia triacanthos* L. In: Silvics of North America. V. 2. Hardwoods. Burns, R. M. and B. Honkala (Tech. Coordinators). Forest Service, United States Department of Agriculture. pp. 358-364.

Burley, J. 1989. Applications of biotechnology in forestry and rural development. In: Applications of biotechnology in forestry and horticulture, Dhawan, V. (ed.), Plenum Press, New York. pp. 9-20.

Chase, S.B. 1947. Propagation of thornless honeylocust. J. For. 45:715-722.

Davis, J.M. 1989. Responses of different genotypes of *Robinia pseudoacacia* L. to micropropagation and *Agrobacterium* - mediated transformation. Ph.D. Thesis, Michigan State University, East Lansing, Michigan. 91 p.

Dirr, M.A. 1977. Manual of woody landscape plants: their identification, ornamental characteristics, culture, propagation and uses. Stipes Publishing Co., Illinois. pp. 203-204.

Dupraz, C. and C. Baldy. 1994. Temperate agroforestry research at INRA, Montpellier, France. Paper presented at the Third North American Agroforestry Conference, August 15-18, 1993. In: Opportunities for Agroforestry in the Temperate Zone Worldwide. Schultz, R. C. and J. P. Colletti (eds.), Ames, Iowa. pp. 445-449.

Fillatti, J.J., J. Sellmer, B. McCown, B. Haissig, and L. Comai. 1987. *Agrobacterium* - mediated transformation and regeneration of *Populus*. Mol. Gen. Genet. 206:192-199.

Funk, D.T. 1957. Silvical characteristics of the honeylocust. U.S. Forest Serv. Central States Forest Expt. Sta. Misc. Release 23. 14 pp.

Gold, M.A. 1984. Honeylocust (*Gleditsia triacanthos* L.): Genetic variation and potential use as an agroforestry species. Ph.D. Thesis, Michigan State University, East Lansing, Michigan. 163 p.

Han, K. 1991. *Agrobacterium rhizogenes* - mediated transformation and plant regeneration from normal transgenic tissues of *Robinia pseudoacacia* L. Ph. D. Thesis, Michigan State University, East Lansing, Michigan. 96 p.

Heit, C. E. 1967. Part 6: Hardseededness - a critical factor. Amer. Nurseryman. 125:10-12, 88-96.

Huxley, P.A. 1983a. Some characteristics of trees to be considered in agroforestry. In: Plant Research and Agroforestry, Huxley, P. A. (ed.), ICRAF P.O. Box 30677, Nairobi, Kenya. pp. 3-12.

Huxley, P.A. 1983b. The role of trees in agroforestry: Some comments: In: Plant Research and Agroforestry, Huxley, P.A. (ed.), ICRAF P.O. Box 30677, Nairobi, Kenya. pp. 257-270.

Robertson, K.R. and Y.T. Lee. 1976. The genera of Caesalpinoideae (Leguminosae) in the Southeastern United States. J. Arn. Arb. 57 (l):1-53.

Rogozinska, J.H. 1967. Triacanthine, growth substances and the *in vitro* culture of *Gleditsia triacanthos* L. Bull. de L'Academie Polonaise des Sciences Cl. II.- Vol. XV, No. 6. pp. 313-317.

Rogozinska, J.H. 1968. The influence of growth substances on the organogenesis of Honeylocust shoots. (In Polish). Acta. Soc. Bot. Pol., 37:485.

Rogozinska, J. H. 1969. Organogenetic differentiation in honeylocust cotyledons. Bull. de L'Academie Polonaise des Sciences Cl.II. Vol. XVII, No. 11-12. pp. 721-725.

Santamour, F. S. Jr. 1978. *Where are the sweet honeylocust today*? AABGA Bull. 12(1):24-28.

Smith, J.R. 1950. Tree crops. A permanent agriculture. The Devin-Adair Company, New York. pp. 65-79.

Srivastava, H.K. 1989. Genetic engineering of tree species. In: Applications of Biotechnology in Forestry and Horticulture, Dhawan, V. (ed.), Plenum Press, New York. pp. 323-330.

Stoutemeyer, V.T., F.L. O'Rourke, and W.W. Steiner. 1944. Some observations on the vegetative propagation of honeylocust. J. Forestry 42: 32-36.

Williams, G. 1982. Energy conserving perennial agriculture for marginal land in southern Appalachia. Final Tech. Report to Dept. of Energy Appropriate Technology Small Grants Program. 37 p.

CHAPTER 1

Shoot regeneration from young and mature plants of *Gleditsia triacanthos* L.

ABSTRACT

The response to tissue culture of various types of honeylocust explants, including cotyledon and hypocotyl explants from 5 to 6 day-old sterile germinating seedlings, nodal explants from 8 and 10 month-old seedlings, root suckers, stump sprouts and elongating summer crown shoots, and winter dormant buds was studied.

Cotyledon and hypocotyl explants that formed callus when cultured on MS medium supplemented with various BAP levels (5-20 μM) were induced to form shoot buds. Elongation was promoted by transfer to lower BAP level. MS medium supplemented with BAP promoted bud emergence and elongation of buds in nodes of 8 and 10 month-old seedlings. There was a differential response of buds in various nodal positions along the stem axis - those closer to the root were the least responsive.

Winter dormant buds from ornamental trees and trees in a provenance-progeny test failed to elongate under any treatment. This failure led to attempts to identify other more responsive explants from mature trees. Buds from nodal segments of root suckers and stump sprouts elongated in modified MS media containing reduced nitrates and supplemented with BAP, zeatin, NAA and gibberellic acid. Leaf yellowing of elongated shoots and late appearance of contamination in elongated shoots were problems that reduced the number of healthy, micropropagated shoots.

Protocols from this research may be important for use in a culture system for propagating elite genotypes which are selected for agroforestry or for ornamental use.

INTRODUCTION

Gleditsia triacanthos L. (honeylocust), a leguminous tree/shrub grows naturally in temperate and subtropic North and South America and Asia (Allen and Allen, 1981; Robertson and Lee, 1976). Some clones, such as those of the thornless cultivar "inermis," are used for landscape planting in the United States and Canada. The cultivar "Sunburst" is widely planted for its vivid yellow foliage in early spring and its yellowish green summer hue (Barnes and Wagner, 1981). Gold and Hanover (1993) mentioned that honeylocust is an ideal multipurpose tree. It possesses a combination of desirable traits which include high wood specific gravity, abundant coppicing, tap-rooted/profusely branched root system, drought tolerance, high carbohydrate percentage in pods and a high protein concentration in the seeds and leaves. Pods are a valuable supplemental livestock feed for silvopastoral systems (Smith, 1950). Recent biotechnologies could offer possibilities for achieving both the development and improvement of honeylocust cultivars for use in agroforestry or as ornamentals.

Micropropagation could be used as a fast method of multiplying genetically superior honeylocust seed or seedling selections for planting in forests, agroforestry schemes, or in seed orchards (Aitken-Christie, 1984). Juvenile materials are very responsive to tissue culture and are good sources of sterile materials. Moreover, plant regeneration from cotyledons and hypocotyls of germinating seeds could be used for the multiplication of plantlets from seeds of selected trees, or hybrid trees, and in cases where seeds of a species are rare, difficult to collect and/or expensive (Fossard *et al.*, 1978). Also, since tree species typically rely on cross-pollination instead of self-pollination, much genetic variation exists in any seed population (Thorpe *et al.*,

1991). When clonal propagation is done, the existing genetic variation is readily made available for breeding. In addition, techniques using juvenile materials will help insure the constant availability of selected seedlings, especially during poor harvest seasons.

Also, for mature trees vegetative propagation has several advantages. The importance of developing culture systems to propagate unique individuals that have desirable traits and the relevance of these culture systems in increasing the efficiency of a breeding program have been discussed (Keathley, 1984; Bonga, 1982; Dunstan and Thorpe, 1986; Franclet, 1983). Plantlet formation via organogenesis in callus cultures has been the route for regeneration of some woody plants (Thorpe, *et al.*, 1991). More recently, organ cultures such as axillary bud culture has been useful in multiplying mature selected angiosperms (Thorpe, 1990; Thorpe *et al.*, 1991; Chalupa, 1983).

The major problems encountered in micropropagation of mature woody plants are bacterial and fungal contamination and the secretion of oxidized polyphenols and tannins into the medium by explants. Approaches have been developed to minimize the damage caused by these factors (George and Sherrington, 1984; Debergh and Read, 1991). In addition, by the time trees are old enough for evaluation, they are often recalcitrant in culture (Thorpe *et al.*, 1991). The juvenility-maturity problem has led to the identification of more suitable sources of sterile explants and techniques for rejuvenating parts of the donor tree (Bonga, 1987; Chalupa, 1983).

Studies by Rogozinska (1967, 1968 and 1969) were the first reports of honeylocust tissue culture. Using a factorial experimental design, Rogozinska (1969)

showed a synergistic interaction between naphthalene acetic acid (NAA) and benzylaminopurine (BAP) and found that on excised cotyledons shoot bud induction was stimulated by high BAP levels. A low concentration of this cytokinin in the presence of NAA stimulated root induction. In these studies, shoot and root regeneration were successfully induced on isolated cotyledon disks. Using one-year shoots from an 80 year-old honeylocust tree, callus formation was induced as well as shoot and roots (Rogozinska, 1968). These studies, however, did not mention regeneration of plantlets with integrated root-shoot axes and the acclimatization process for outplanting.

Work was also done at the University of Georgia School of Forest Resources (Sommer, personal communication) on tissue culture of honeylocust seeds as part of a project on screening a series of several hardwoods. Cotyledons were used as the explant. Callus formation was observed and adventitious shoots were regenerated from this callus although it was a sporadic event. Thus, a protocol was not developed. There was an instance of a culture that produced embryoids from the cotyledons through callus. It was expected that repeating such experiments with immature embryos could have been more successful. Shoot tips of seedlings were also tried (Sommer, unpublished observations). Shoot and root regeneration were achieved in cultures of nodal explants of 4 week-old sterile seedlings (Basbaa *et al.*, 1993).

This chapter reports studies of the response to tissue culture of seedling explants, dormant buds from crown shoots, nodal explants from 8 and 10 month-old-seedlings, elongating branched shoots, stump sprouts and root suckers. The extent of

contamination was examined for each explant and bud break and shoot elongation were induced.

The goals of the experiments were: (1) to obtain shoot regeneration on cotyledon and hypocotyl segments of germinating seeds, and nodal explants of 8 and 10 month-old seedlings, and (2) to identify responsive explant tissues from juvenile and mature trees for axillary bud elongation.

MATERIALS AND METHODS

A. Germinating seedlings

a. Seed source. Seeds of honeylocust (Figure 1A) were obtained from the MSU Tree Research Center where seed collections are stored at 4°C. These seeds were part of the collections used in a genetic variation study (Gold, 1984). The seed accession numbers were: 4333-0168, 4333-0395 and 4333-0431 corresponding to three half-sib open-pollinated families. These seed collections were made from trees growing in Huntingdon, Pennsylvania; Corson, South Dakota; and Tompkins, New York respectively.

b. Seed scarification and sterilization. The seeds were scarified prior to germination using a thirty minute soak in concentrated sulfuric acid (Heit, 1967). The seeds were then rinsed under running tap water for several minutes. The seeds were sterilized by soaking in 1.0% sodium hypochlorite solution containing 10 drops/l Tween 20 surfactant for 15 minutes. The seeds were rinsed 4-5 times with sterilized double-distilled water and allowed to stand in the last rinse for 30 minutes. They were then rinsed twice in water and transferred to germination medium.

c. Seed germination. The seeds were placed individually in 10 x 150 mm test tubes each containing 5 ml of agar media with 5 g/l sucrose and 8 g/l agar (Difco bacto-agar) for germination. After 5-6 days, percent germination was scored in each of the five seed lots initially tested. The three seed lots giving the highest percent germination (52.5%, 80%, and 100%) were used for tissue culture.

d. Explant source. From the seedlings (Figure 1B) of the three open-pollinated families, 10 mm-long hypocotyl segments were excised with a sterilized scalpel and 0.6 mm diameter cotyledonary disks (Figure 1C) were excised with a 0.6 mm hole punch.

e. Culture conditions. MS (Murashige and Skoog, 1962) medium containing 8 g/l sucrose and 0.8% agar was supplemented with four levels of BAP (5.0 μM, 10.0 μM, 15.0 μM and 30.0 μM) and 1 μM NAA. Seeds of the three families were tested in all treatments using five hypocotyl explants and 5 cotyledon explants from each of 6 seedlings per treatment. Cultures were subcultured 3x in the same medium at three week intervals prior to data collection. All cultures were maintained in controlled environment chamber at 26-27°C with an 18 hr. light/6 hr. dark lighting regime (30-50 μM/s/m^{2}PAR) using a cool fluorescent white lamp (F96T12/CW/VHO).

f. Characters scored and analysis of data. Cultures were scored for callus initiation, shoot and root formation. Mean frequencies of callus, shoot and root formation were computed. An analysis of variance was performed for a factorial experiment in a completely randomized design (3 families x 4 BAP levels x 2 explant

types) using Systat software (Wilkinson, *et al.*, 1992). Frequency data were transformed using arcsin transformation prior to analysis (Steel and Torrie, 1980).

g. Shoot elongation and maintenance of cultures. Primary cotyledon explants with emerging shoots were divided into 2-3 pieces and transferred to sterile glass jars (55 x 72 mm) each containing 20 ml of MS medium supplemented with 20 g/l sucrose and 1 μM BAP and medium was solidified with 0.8% agar. Hypocotyl segments with emerging shoots were excised from the primary explants and also subcultured onto this medium. Elongating shoots were maintained singly for 2-3 months in 18 x 150 mm test tubes each containing 10 ml of MS medium plus 0.5 μM BAP before initiating the rooting experiments.

B. Eight and ten month-old seedlings

a. Explant source. Seeds of the Calhoun source were germinated in paper pots containing a peat:perlite:vermiculite mix (1:1:1) in the growth chamber with a light intensity of 70-100 μM/s/m^{2}PAR. Calhoun is one of two outstanding clones bearing high-quality pods discovered by the Tennesee Valley Authority (Chase, 1947). In twenty 10 month-old seedlings, nodal segments (ca. 2.0 cm in length, bearing a part of the petiole and segments of the internode on both ends) were excised starting with the second node above the cotyledonary node and continuing until the 3rd or 4th node below the shoot apex. For each of 24 ten month-old seedlings, from the cotyledonary node, the second, fourth, sixth, eighth, tenth, twelfth, fourteenth and sixteenth nodal segments toward the apex were excised with a pruner.

b. Sterilization. Nodal segments were soaked in 30% Liquinox detergent solution for 20-30 min. and washed under running water. In the laminar flow hood

the explants were dipped in 70% ethyl alcohol for 2 minutes and washed with sterile double distilled water (ddw) before treating them with 1% sodium hypochlorite solution containing 10 drops/l of Tween-20 surfactant for 30 minutes, washing them 4x with sterile water, and then allowing them to stand for 20 minutes in sterile water before the final rinse in ddw. The cut ends of each nodal explant were further trimmed with a sterilized scalpel with ca. 1 cm of the petiole left intact.

c. Culture medium. Modified MS medium with half strength of NH_4NO_3 and KNO_3 plus 20 g/l sucrose was used. Casamino acid at 250 mg/l was also added to the medium. For explants from 8 month-old seedlings, the MS medium contained 1 μM or 0 of each of BAP and zeatin and 0.2 μM or 0 of gibberellic acid (GA) in a factorial combination. The medium was adjusted to pH 5.8 and solidified with 8 g/l agar. Zeatin and gibberellic acid were filter (0.22 μm Millipore)-sterilized. Twenty-four explants were cultured per treatment. For explants from 10 month-old seedlings, the medium contained modified MS medium with 1 μM BAP. This was chosen from among the eight treatments used for 8 month-old seedlings. Twenty-four explants were used per node position. In both experiments, one explant was cultured per tube.

d. Characters scored and data analysis. Frequency of contamination was scored at day five. Bud elongation was scored at day 21. Data on frequencies of bud elongation from 8 month-old seedlings were analyzed using standard statistical procedure for analysis of variance for a factorial experiment (2 BAP levels x 2 zeatin levels x 2 GA levels) in a completely randomized design using Systat software (Wilkinson *et al.*, 1992). Frequency data were transformed using arcsin transformation prior to analysis. An analysis of variance was performed on data for

frequency of bud elongation from 10 month-seedlings as in the previous experiment but for a single factor. Pairwise contrasts between node positions were analyzed using multiple comparisons based on Kruskal-Wallis rank test (Conover, 1971; Hollander and Wolfe, 1973) by the use of Systat software (Wilkinson *et al.*, 1992).

C. Juvenile and mature trees. The explant sources are shown in Table 1. Dormant buds were collected from branched shoots of ornamental honeylocust trees planted in the MSU campus and also from trees planted in Sandhill that were in a provenance/progeny test (Gold, 1984). Elongating shoots in summer were also collected from Sandhill trees. Shoots were collected from root suckers (Figure 3A) of three selected trees and stump sprouts were taken from two stumps. All stem cuttings were stored temporarily on ice during transport to the laboratory. Cuttings were stored at 8°C until used.

Table 1. Plant materials cultured for juvenile and mature tree propagation.

Explant	Place of collection	No. of trees		Approx. age	Date of collection
		Juvenile	Mature		
dormant buds	Spartan Village		4	36	Jan. -Feb. 1991
	Cherry Lane		4	37	Dec. 1991, Jan. 1991
	Sandhill	9		14	April 1991
nodal segments from root suckers	MSU Library		2	38	July 1991, 1992
	Spartan Village		1	36	August, October 1991
	Cherry Lane		1	37	August, October 1991
	Wilson Hall		1	27	August, October 1992
	Urban Planning		1	26	August 1991
summer crown shoots	Sandhill	3		14	August 1992
nodal segments from stump sprouts	Grand River Avenue		1(?)	-	Aug., Sept. 1992
	Mount Hope		1(?)	-	Aug., Sept. 1992

?= no available information if tree had flowered

1. Bud Culture

1.1 Ornamental trees

a. Explant source. Bud cultures were initiated using pre-sterilized stem segments (Figure 2A). The honeylocust bud is enveloped by overlapping bud scales with as many as five buds found in a single node (Figures 2B & C). Thus, the

following kinds of bud explants was used: (1) single buds (Figure 2D) as the two largest buds from each node. In some experiments, buds with outer bud scales excised and inner ones intact were tested, while in other experiments a bud with all bud scales excised was used; (2) aggregate buds as all buds in a node with scales partly removed.

b. Sterilization. Stem segments (3-5 cm in length) with one node were surface sterilized as described for 8 and 10 month-old seedlings except that a higher concentration of sodium hypochlorite (2.5%) was used. Given the relative effectiveness of the sterilization procedures in the first two experiments, the same procedures were adopted in succeeding bud culture experiments and shoot culture experiments.

c. Culture medium and maintenance of cultures. MS medium was used in bud culture experiments. In an effort to induce development of the dormant buds, some components added to the basic MS medium were varied. These were carbon source, agar concentration, cytokinin types and the levels tested. These are described in the respective section of each experiment. Bud leaves that remained green or that turned brown were scored and greenish cultures were subcultured and maintained for one year.

Experiment 1. Single buds with bud scales excised and buds with only outer scales excised were used as explants. Buds came from one tree. MS medium contained 20 g/l sucrose, supplemented with 4 levels of BAP (μM): 0.1, 0.32, 1.00 and 3.2 and 0.5 μM NAA and solidified with 8 g/l Bacto-agar. Twenty buds were tested per treatment; four buds in each of five Petri dishes (100 x 15 mm)

each containing 25 ml of the medium. Petri dishes containing the buds from each treatment were blocked inside the growth chamber.

Experiment 2. All the buds in each node as an aggregate were excised and used as an explant. MS medium was used with NAA, sucrose, and agar in concentrations as in the previous experiments. The BAP levels (μM) were 1.0, 3.2 and 5.0. Twenty bud aggregate explants were cultured per treatment, four bud aggregates in each of 5 Petri dishes. Petri dishes containing the buds were blocked as in the previous experiment.

Experiment 3. Single buds with inner scales intact were used. Two kinds of carbon source: 20 g/l sucrose and 20 g/l fructose, BAP levels (1.0 μM, 3.20 μM and 5.0 μM) and NAA levels (0 and 0.5 μM) were tested in a factorial combination in blocks. The media were solidified in 0.8% agar.

Experiment 4. Single bud explants with inner scales intact were used as explants. MS medium containing sucrose was used. Two agar concentrations (0.6% and 0.8%), BAP and NAA levels were tested in a factorial combination as in the previous experiment. Other cultural conditions were as in Experiment 3. Twenty dissected buds were cultured per treatment in Petri dishes as described in previous experiments.

Experiment 5. Single bud explants with inner scales intact were used. The MS medium contained 20 g/l sucrose and solidified by 0.8% agar. Three levels of zeatin were tested: 5×10^{-7} μM, 0.10 μM, and 1.0 μM. Twenty single buds were cultured per treatment in Petri dishes as in previous sections.

d. Characters scored and data analysis. Contamination of explants was scored 5 days after culture initiation and uncontaminated explants were subcultured and maintained on the same medium. Bud leaf coloration (green or brown) frequency was scored per Petri dish initially consisting of 4 explants on the third week under the stereoscopic microscope. In aggregate bud culture (Expt. 2), the explant was scored green if at least one bud was green. Frequency data of Experiments 1-5 were transformed using the arcsin transformation for proportions. Data of Experiments 1-4 were used for analysis of variance for randomized complete block design. Treatment factors found significant were further analyzed by Friedman's two-way analysis of variance by ranks (Conover, 1971; Hollander and Wolfe, 1972). An analysis of variance for completely randomized design was performed on data from Experiment 5. All statistical analysis were performed using Systat software (Wilkinson *et al.*, 1992) as in the previous experiments.

1.2 Trees from a provenance/progeny test

a. Explant source. Dormant buds were collected from 3 tall, 3 intermediate and 3 short families as defined for that plantation by Gold (1984). These are three height classes found in a rangewide provenance/progeny test (Gold, 1984). Buds with outer scales excised were used for culture.

b. Sterilization. Sterilization and bud excision were as described in buds from ornamental trees.

c. Culture medium and maintenance of cultures. MS medium containing 20 g/l sucrose was supplemented with 3.2 μM BAP and 0.5 μM NAA. Forty buds were cultured from each of the trees.

d. Characters scored and data analysis. Contamination was scored after 5-6 days and callus formation from the remaining aseptic explants were scored after three months. Contamination and callus formation percentages were computed for each genotype.

2. Shoot Culture

a. Explants. Shoot cultures were initiated with root sucker explants (Figure 3A), summer crown shoots and stump sprouts (Figure 3B). Collection locations are given in Table 1. Explants used were nodal segments (Figure 3C) approximately 2.0-2.5 cm long bearing the axillary bud with a small segment of the petiole that protects it and a small part of the internode on both ends of the node. The nodal explant used was similar with those of 10 and 12 month-old seedlings.

b. Sterilization. Surface sterilization methods were as stated for bud cultures from mature trees.

c. Culture media. Modified MS medium was used as in nodal cultures of 8 and 10 month-old seedlings. The phytohormones tested are stated below for each type of explant.

c.1 Root suckers

Experiment 1. (August 1991 collections). MS medium was supplemented with BAP, zeatin, naphthalene acetic (NAA) and gibberellic acid (GA). Three treatments were tested: (1) 1 μM BAP, 1 μM zeatin, 0.5 μM NAA and 0.1 μM GA, (2) 1 μM BAP, 1 μM zeatin, 0.5 μM NAA and 0.3 μM GA, (3) 1 μM BAP, 1 μM zeatin, 0.5 μM NAA and 1.5 μM GA. Zeatin and GA were filter-sterilized (0.22 μm Millipore filter unit) and added to the partially-cooled (ca. 45-50°C) autoclaved

medium. Twenty ml of the medium was poured into each Petri dish (100 x 15 mm). Thirty explants were cultured per treatment, three explants in each of 10 Petri dishes. After scoring for contamination, aseptic explants were subcultured in test tubes (20 x 150 mm) each containing 10 ml of the same medium as in the initial cultures. Three rootstock genotypes were tested.

Experiment 2. In October 1991 root suckers were collected from two of the same trees tested in August 1991. There was no re-growth of root suckers in the third genotype. Due to a limited amount of available root suckers from the trees, only two treatments were repeated. These were: 1) 1 μM BAP, 1 μM zeatin, 0.5 μM NAA and 0.1 μM GA, (2) 1 μM BAP, 1 μM zeatin, 0.5 μM NAA and 0.3 μM GA. Forty explants were cultured per treatment in 10 ml of media in each of 10 x 150 mm test tubes. To produce more shoots for rooting experiments root suckers were collected from rootstocks of two other ornamental trees in the MSU campus in 1992. The same treatments were tested as in the preceding experiments. Elongating cultures including those with yellowing leaflets were subcultured on the same MS medium used initially but was supplemented with only 1 μM BAP. Cultures were maintained in this medium until they were used for rooting experiments.

c.2 Stump sprouts and summer crown shoots. Five treatments were tested, each one containing 0.5 μM NAA and varying BAP concentrations (μM): 0.1, 0.5, 1.0, 5.0, and 10.0. Twenty-four explants were cultured per treatment in 10 ml of medium in each test tube (20 x 150 mm). Sprouts from two tree stumps were tested. Explants were collected from summer shoots of three trees at Sandhill.

d. Characters scored and data analysis

d.1 Root suckers. Frequency of explant contamination after 5 days, bud break (petiole dehisced and emerging bud is evident at the axil after 15 days), shoot elongation (elongated young shoot with shoot apex visible) after 21 days in bud breaking explants, yellowing and dehiscence of leaves in elongated shoots in culture, contamination after 30 days were scored and shoot length after 60 days was measured. Aseptic explants were scored for the occurrence of budbreak. Data from these explants were analyzed by the standard analysis of variance for completely randomized designs. If any treatment factor was found significant for a character measured, the data were also analyzed by Kruskal-Wallis rank test (for unequal samples) for the three treatments tested (differing in GA concentration). Replicates of four explants each were used for analysis. Replicates with contaminated explants were not included in the analysis. Treatments 1 (0.1 μM GA) , 2 (0.3 μM GA) and 3 (1.5 μM GA) had 14, 11, and 12 replicates, respectively. Analysis of variance was not carried out on bud elongation, shoot yellowing and shoot length data due to small number of samples, which resulted from the low number of initial explants which developed to a size sufficient for use in this experiment.

d.2. Stump sprouts and summer crown shoots. Contamination, bud break and elongation frequencies were scored. Budbreak frequency data were analyzed as previously stated for experiments involving root suckers. Analysis of variance on data from cultures of nodal explants from crown shoots was not carried out due to the small and widely varying number of remaining samples in the various treatments after contaminated explants were discarded.

RESULTS

A. Germinating seedlings. All seedling explants produced callus (Figures 1D & E) in all treatments. Callus initiation was typically evident after 7-10 days in culture. The callus varied in color from white to light green. Callus was formed at the cut edges of cotyledons and on cut ends of hypocotyls including surfaces directly in contact with the medium. In addition, shoots were formed in all BAP levels tested (Table 2), but they occurred at lower frequencies than callus. Shoots (Figures 1F & G) were formed in areas where callus also formed. Shoot formation was preceded by green nodular structures (shoot buds) in the callus, evident after one month in culture. Elongation was promoted by transfer to 1 μM BAP (Figure 1H).

Shoot formation was significantly different between treatments and between families (Tables 2 & 3). Mean percentage of shoot induction increased with increasing BAP levels. Roots were observed only in cotyledon explants, not in hypocotyl explants. Root formation was also significantly different between families (Tables 4 & 5).

Table 2. Shoot formation in cotyledon and hypocotyl explants on MS medium containing varying levels of BAP .

	Shoot formation (%)[1)]						
	Family 1		Family 2		Family 3		
	Cotyl.	Hypo.	Cotyl.	Hypo.	Cotyl.	Hypo.	
BAP (μM)							Mean %*
5	0	3	0	0	0	0	0.5 [a]
10	0	7	0	7	0	0	2.3 [a]
15	10	27	23	10	47	33	25.0 [b]
30	58	63	57	3	88	20	48.0 [c]

Averages:

Family** Family = 21[b] Family 2 =12[a] Family 3=24[b]

Explant Hypocotyl =14[ns] Cotyledon =24[ns]

1) average percentage of 6 replications, each with 5 explants

*significant at $\alpha_{0.05}$

** significant at $\alpha_{0.01}$

ns=not significant. Numbers followed by different letters are significantly different (Tukey's test).

Table 3. ANOVA of shoot formation in cotyledon and hypocotyl explants on MS medium containing varying levels of BAP.

Source	SS	df	MS	F-ratio
Treatment (BAP)	41673.592	3	13891.197	15.7962*
Explant	1598.267	1	1598.267	0.713ns
Family	2167.414	2	1083.707	4.750**
Treatment x explant	8889.532	3	2963.177	5.25**
Treatment x family	5276.387	6	879.398	3.854**
Explant x family	4482.037	2	2241.019	9.822**
Treatment x explant x family	3385.327	6	564.221	2.473*
Error	27379.625	120	228.164	

** = significant at 1% level, * = significant at 5% level, ns = not significant

Table 4. Root formation in cotyledon and hypocotyl segment explants on MS medium containing varying levels of BAP.

	Root formation (%)[1]						
	Family 1		Family 2		Family 3		
	Cotyl.	Hypo.	Cotyl.	Hypo.	Cotyl.	Hypo.	
BAP levels							Average[ns]
5	0	0	7	0	13	0	3
10	3	0	20	0	3	0	4
15	0	0	3	0	30	0	6
30	0	0	0	0	7	0	1
Averages:							
Family*	Family 1 = 0.4[a]		Family 2 = 4[ab]		Family = 7[b]		
Explant[ns]	Cotyledon = 7[ns]		Hypocotyl = 0[ns]				

[1] Average of 6 replications, each with 5 explants.
*significant at $\alpha_{0.05}$, ns = not significant; numbers followed by different letters are significantly different (Tukey's test)

Table 5. ANOVA of root formation in cotyledon and hypocotyl explants in medium containing varying levels of BAP.

Source	SS	df	MS	F-ratio
Treatment	307.645	3	102.548	0.477^{ns}
Explant	2194.610	1	2194.610	4.630^{ns}
Family	946.864	2	473.432	5.051*
Treatment x explant	307.645	3	102.548	0.477^{ns}
Treatment x family	1288.324	6	214.721	2.291*
Explant x family	946.864	2	473.432	5.051*
Treatment x explant x family	1288.324	6	214.721	2.291*
Error	11248.120	120	93.734	

** = significant at 1% level, * = significant at 5% level, ns = not significant

B. Eight and ten month-old seedlings. Contamination was 4% (7 contaminated out of 192 total sample explants) in the 8 month-old seedlings and 3% (5 contaminated explants out of 192 total samples) in the 10 month-old seedlings. Effects among BAP levels and GA levels were significantly different (Table 6 & 7). The MS medium plus 1 μM BAP alone produced the highest percentage of emerged shoots among all treatments (Tables 6). Frequency of bud elongation was significantly higher at 1.0 μM BAP (58%) than without BAP (30%) across all levels of zeatin and gibberellic acid. Absence of GA in the medium was significantly higher (51%) than presence of 0.2 μM GA (37%) across all levels of BAP and zeatin (Tables 6). Zeatin effects were not different between the two levels tested.

Table 6. Bud elongation of nodal explants from 8 month-old honeylocust seedlings. Levels of the phytohormones are in μM. Each treatment (BAP/zeatin/GA combination) has n=24 initially.

	bud elongation (%)[a]				
	0 GA_3		0.2 GA_3		
	Zeatin				
BAP	0	1	0	1	BAP averages*
0	17(24)	50(24)	18(22)	33(24)	30
1	86(21)	50(24)	44(23)	52(23)	58
Averages:					
GA_3*	0 GA_3=51*		0.2 GA_3=37*		
Zeatin	0 zeatin=41[ns]		1 zeatin=46[ns]		

[a] values in parenthesis refer to no. of remaining aseptic explants
* significant at $\alpha_{0.05}$ (Table 7)
ns=not significant

Table 7. ANOVA of the response of nodal explants (bud elongation) from 8 month-old honeylocust seedlings to tissue culture.

Source	SS	df	MS	F-ratio
BAP	3.695	1	3.695	17.256**
Zeatin	0.133	1	0.133	0.621ns
GA_3	0.879	1	0.879	4.106*
BAP x Zeatin	1.644	1	1.644	7.679**
BAP x GA_3	0.179	1	0.179	0.836ns
Zeatin x GA_3	0.198	1	0.198	0.927ns
BAP x Zeatin x GA_3	1.130	1	1.130	5.277*
Error	37.902	177	0.214	

** = significant at 1% level, * = significant at 5% level, ns = not significant

Bud elongation (%) of explants from the different node positions varied significantly (Table 8). It was highest (91%) in explants from node position 8. This frequency was significantly higher than those from node positions 2 and 4 (Kruskal-Wallis rank test, Tables 9 & 10).

Table 8. ANOVA of the bud elongation of explants from different node positions in 10 month-old honeylocust seedlings.

Source	SS	df	MS	F-ratio
node	11.149	7	1.593	8.234**
Error	34.626	179	0.193	

** = significant at 1% level

Table 9. Bud elongation frequencies of explants from different node positions of 10 month-old honeylocust seedlings in MS medium supplemented with 1 μM BAP.

Node position	Replications[1)]	Mean % bud elongation[2)]	Rank mean*
2	5	25[a]	10.40
4	6	25[a]	10.83
6	5	50[ab]	19.10
8	5	88[b]	34.70
10	6	79[ab]	30.58
12	6	79[ab]	30.58
14	5	70[ab]	26.30
16	6	46[ab]	17.58

[1)] each replication has 4 explants

[2)] average of the replications, significant by Kruskal-Wallis test at 5% level (Table 10), means with common letter are not significantly different.

* significant by Kruskal-Wallis test ($k-1=7$, $\alpha_{0.05,}$), Kruskal-Wallis test statistic=21.40, higher than tabular value= 14.07.

Table 10. Multiple comparison of bud elongation frequencies from different node positions of 10 month-old honeylocust seedlings based on Kruskal-Wallis rank sums test. Differences in rank means between node positions are compared with the critical value at $\alpha_{0.1}$ (*= greater than critical value= 23.69, [b]=greater than critical value=22.64).

Node	2	4	6	8	10	12	14	16
2		0.43	8.70	24.30*	20.18	20.18	15.90	7.18
4			8.27	23.87[b]	19.75	19.75	15.47	6.75
6				15.60	11.48	11.48	7.20	1.52
8					4.12	4.12	8.40	17.12
10						0	4.28	13.0
12							4.28	13.0
14								8.72
16								

C. Juvenile and Mature trees

1. Bud culture

a. Ornamental trees. Contamination in cultures of bud explants from a single tree was 20% (16/80) with the bud scales left intact and 18% with the scales removed (14/80). Frequency of green buds did not differ significantly between those with scales and those without scales (Tables 11 & 12). Green buds were observed in all levels of BAP tested. The frequency of green buds was not significantly different among the BAP levels (Tables 11 & 12).

Table 11. Test of explant type and BAP levels on the early response (greenness of young leaves) of dormant buds in tissue culture.

	Greenness (%)[a]				
	BAP levels (μM)				
	0.10	0.32	1.0	3.2	
Explant type					Explant averages[b]
Bud with scales	25	25	40	40	32
Bud without bud scales	25	56	45	35	40
BAP averages[b]	25	40	42	37	

[a] each treatment (explant type/BAP level combination) has 5 Petri dishes, each with 4 buds.
[b] not significant, (ANOVA, Table 12)

Table 12. ANOVA of explant type and BAP levels on the early response (greenness of young leaves) of dormant buds cultured *in vitro*.

Source	SS	df	MS	F-ratio
Block	1203.750	4	300.938	1.176^{ns}
Bud	455.625	1	455.625	1.780^{ns}
BAP	1051.875	3	350.625	1.370^{ns}
Bud x BAP	466.875	3	155.625	0.608^{ns}
Error	7166.250	28	255.938	

^{ns} = not significant

The percentages (73%, 83% and 75% in 1.0 μM BAP, 3.2 μM BAP and 5.0 μM BAP respectively), of bud greenness in aggregate bud culture was not significantly different among the three BAP levels (ANOVA, Table 13).

Table 13. ANOVA of the early response (greenness of leaves) of aggregate dormant buds to BAP levels.

Source	SS	df	MS	F-ratio
Block	660.00	5	132.00	0.940^{ns}
BAP levels	202.50	2	101.25	0.845^{ns}
Error	4110.00	7	587.143	

^{ns} = not significant

Carbon source (fructose vs. sucrose) and bacto-agar concentration (0.6% vs. 0.8%) did not significantly affect explant coloration (Tables 14, 15, 16 & 17). Frequency of green buds was significantly different among the three BAP levels (ANOVA in Tables 16 & 17). In these experiments, green frequencies at 1.0 μM BAP and 3.2 μM BAP were significantly higher than at 5.0 μM BAP across levels of NAA (Table 14) or agar concentration (Tables 15). The effect of NAA, and interactions, i.e. carbon X BAP, carbon X NAA, BAP X NAA and carbon X BAP X NAA were significant (Table 16). These green buds did not show elongation.

Table 14. Response of dormant honeylocust buds to BAP, NAA and to fructose and sucrose. Levels of phytohormones are in μM.

	mean greenness (%)[1)]				
	fructose (20 g/l)		sucrose (20 g/l)		
BAP	0 NAA	0.5 NAA	0 NAA	0.5 NAA	BAP averages*
1.0	70	20	85	55	58 ^{b}
3.2	75	30	48	70	56 ^{b}
5.0	30	40	15	15	25 ^{a}
Averages: fructose	fructose= 44^{ns}		sucrose=48^{ns}		
NAA*	0 NAA= 54^{a}		0.5 NAA=38^{b}		

[1)] from each treatment (BAP/carbon/NAA) = 5 plates with 4 buds each. Plates with all four buds contaminated were not included.
* standard ANOVA significant at $\alpha_{0.01}$, $P<0.05$ (Table 16). Averages with different letters are significantly different (Friedman two-way ANOVA, Appendix 1).
ns= not significant

Table 15. Response of dormant honeylocust buds to MS medium solidified by two concentrations of bacto-agar, NAA and BAP. Levels of phytohormones are in μM.

	Mean greenness (%)[1)]				
	0.6% bacto-agar		0.8% bacto-agar		
BAP	0 NAA	0.5 NAA	0 NAA	0.5 NAA	BAP averages*
1.0	80	40	80	60	65 ^{b}
3.2	55	35	65	60	54 ^{b}
5.0	20	25	10	15	18 ^{a}

Averages:		
Bacto-agar	0.6% bacto-agar = 42 ^{ns}	0.8% bacto-agar = 48 ^{ns}
NAA	0 NAA= 52^{ns}	0.5 NAA=39^{ns}

[1)] from each treatment (BAP/carbon/NAA) = 5 plates with 4 buds each. Plates with all four buds contaminated were not included.

* standard ANOVA significant at $\alpha_{0.05}$, $P<0.05$ (Table 17). Averages with different letters are significantly different (Friedman two-way ANOVA, Appendix 2).

^{ns} not significant

Table 16. ANOVA of the response of honeylocust dormant buds to MS medium containing varying BAP and NAA levels and carbon (fructose or sucrose) sources.

Source	SS	df	MS	F-ratio
Block	622.500	4	155.625	1.461 ^{ns}
Carbon	33.750	1	33.750	0.317^{ns}
BAP	6577.500	2	73288.750	30.870 **
NAA	1653.750	1	1653.750	15.523**
Carbon x BAP	3532.500	2	1766.250	16.579**
Carbon x NAA	843.750	1	843.750	7.920**
BAP x NAA	2632.500	2	1316.250	12.355**
Carbon x NAA x BAP	1507.500	2	753.750	7.075**
Error	4687.500	44	106.534	

** = significant at 1% level, ^{ns} = not significant

Table 17. ANOVA of the response of honeylocust dormant buds to MS medium containing varying BAP and NAA levels, solidified by two concentrations of bacto-agar.

Source	SS	df	MS	F-ratio
Block	3712.500	4	928.125	1.094^{ns}
Agar	375.000	1	375.000	0.442^{ns}
BAP	19560.000	2	9780.000	11.528**
NAA	1215.000	1	1215.000	1.432^{ns}
Agar x BAP	2190.000	2	1095.000	1.291^{ns}
Agar x NAA	240.000	1	240.000	0.283^{ns}
BAP x NAA	1890.000	2	945.000	1.114^{ns}
Agar x BAP x NAA	390.000	2	195.000	0.230^{ns}
Error	37327.500	44	848.352	

** = significant at 1% level, ^{ns} = not significant

The incorporation of zeatin in MS medium resulted in green frequencies of 88%, 94%, and 90% in the three levels tested which were not significantly different (Table 18).

In most of the cultures, the first leaves of the green buds had unfolded two weeks from start of culture (Figure 2E). The remaining green buds were maintained on the same medium for one year but failed to elongate. Five green buds were found to have enlarged shoot tips as examined under the stereoscope. After 6 months of culture leaves expanded but appeared vitrified (Figure 2F).

Table 18. ANOVA of the response of dormant buds (greenness) to zeatin.

Source	SS	df	MS	F-ratio
Zeatin	211.875	2	105.938	0.152^{ns}
Block	3886.875	4	971.719	1.392^{ns}
Error	4888.125	7	698.304	

^{ns} = not significant

b. Trees from a provenance/progeny trial. There was a widely varied number of aseptic explants among samples from each tree that were recovered (Table 19) after contaminated explants were discarded. Most buds formed callus instead of elongating. Callus started at the basal part of the bud in contact with the medium. This part of the bud enlarged with the proliferation of the callus. Some leaves also expanded but the stem axis did not show elongation and proliferated, covering most of the bud.

Table 19. Callus formation in honeylocust dormant buds of trees from provenance progeny test (Gold, 1984) cultured in MS medium with 3.2 μM BAP and 0.5 μM NAA.

Height class and tree no.	Callus formation[1)] (%)
Short	
1	34/37 (91)
2	0/15 (0)
3	2/8 (25)
Intermediate	
1	30/31 (96)
2	12/12 (100)
3	2/2 (100)
Tall	
1	9/18 (50)
2	3/17 (18)
3	1/12 (8)

[1)]numerator indicates number of callus-forming explants, denominator indicates number of remaining aseptic explants. Percentage callus formation is given in parenthesis. Initial number of explants=40/tree.

2. Shoot culture

a. Root suckers. There was contamination in 41% and 32% of the initial total number of explants cultured from rootstock genotype nos. 1 & 3, and 2 respectively after 5 days (Table 20). Contamination frequencies scored on day 30 were 28% (genotype 1), 27% (genotype 2) and 10% (genotype 3). Budbreak in the nodal explants was evident when the petiole abscised. At the axil of the abscising petiole was the emerging bud. Bud break percentage was significantly different

among the three treatments (GA levels) and not significantly different among the three rootstock genotypes (Tables 21 & 22). Higher budbreak percentages in 0.1 GA and 0.3 GA were significantly different from that in 1.5 μM GA (Kruskal-Wallis rank test, Appendix 3).

Table 20. Percent contamination in nodal explants and regenerated shoots from explants of root suckers of honeylocust.

Rootstock genotype	Contaminated explants at day 5[a] (%)	Contaminated shoots at day 30[b] (%)
1	37/90 (41)	11/39 (28)
2	29/90 (32)	10/36 (27)
3	37/90 (41)	3/30 (10)

[a] the number of contaminated explants scored at day 5 from the start of culture is shown in the numerator, divided by the initial number of explants in the three treatments tested for each rootstock genotype.
[b] the number of contaminated shoots scored at day 30 from the start of culture is given in the numerator, divided by the number of bud breaking explants.

Among those explants where bud break occurred, shoot elongation was also observed (Figure 3D, E & F, Table 23). This occurred in two ways: explants where the shoot elongated (Figure $3F_1$) and explants where only leaves occurred (Figure $3F_2$). The shoot tip was not visible). Yellowing of leaflets of some elongated shoots was also observed in all treatments in each rootstock genotype (Table 24).

Table 21. Percent bud breaking in nodal explants of root suckers. Each treatment contained 1μM BAP, 1 μM zeatin, 0.5 μM NAA and GA.

	Budbreak[1)] (%)			
	Rootstock genotype 1	Rootstock genotype 2	Rootstock genotype 3	
Treatment				Averages*
GA levels (μM)				
0.1	16/22 (73)	13/23 (56)	9/17 (53)	61% ^{b}
0.3	14/16 (88)	15/19 (79)	12/18 (67)	78% ^{b}
1.5	9/15 (60)	8/19 (42)	9/18 (50)	51% ^{a}
Averages	74%^{ns}	59%^{ns}	57%^{ns}	

[1)] the number of explants that showed bud breaking is given in the numerator, divided by the number of uncontaminated explants. Values in parentheses are percentages.
*ANOVA, significant at 0.05 level (Table 22). Values followed by different letters are significantly different by Kruskal-Wallis rank test (α=0.05, Appendix 3).
^{ns} = not significant

Table 22. ANOVA on budbreaking in honeylocust root suckers in three rootstock genotypes (August 1991 collections).

Source	SS	df	MS	F-ratio
genotype	0.867	2	0.433	1.908^{ns}
GA_3	1.950	2	0.975	19.50^{*}
genotype x GA_3	0.201	4	0.050	0.221^{ns}
Error	35.891	158	0.227	

* = significant at 5% level, ^{ns} = not significant

Table 23. Percent shoot elongation in nodal explants of root suckers (August 1991). Each treatment (Tmt) contained 1 μM BAP, 1 μM zeatin and 0.5 μM NAA.

Shoot elongation (%)[1)]

	Rootstock genotype 1	Rootstock genotype 2	Rootstock genotype 3	
Treatment GA (μM)				Averages
0.1	12/16 (75)	11/13 (85)	8/9 (89)	83%
0.3	11/14 (78)	15/15 (100)	10/12 (83)	87%
1.5	7/9 (78)	8/8 (100)	9/9 (100)	93%
Averages	77%	95%	91%	

[1)] the number of elongating explants is indicated in the numerator, divided by the total number of bud breaking explants. Values in parentheses are percentages.

Table 24. Yellowing of elongated shoots in nodal explants of root suckers. Each treatment contained 1 μM BAP, 1 μM zeatin, 0.5 μM NAA and GA.

	Leaf yellowing[1] (%)			
Treatment	Rootstock genotype 1	Rootstock genotype 2	Rootstock genotype 3	Averages
GA levels (μM)				
0.1	5/16 (31)	5/13 (38)	5/9 (55)	41%
0.3	7/14 (50)	7/15 (47)	4/12 (33)	43%
1.5	6/9 (67)	4/8 (50)	1/9 (11)	43%
Averages	49%	45%	33%	

[1] the number of explants with yellowing leaflets is shown in the numerator, divided by the number of bud breaking explants. Values in parentheses are percentages.

The results (Table 25) obtained in nodal explant cultures from regrowth of root suckers from rootstock genotypes 1 & 2 confirmed those obtained in the previous experiment (Table 21), i.e. the two genotypes did not differ significantly in budbreak percentages (Table 26). GA concentrations of 0.1 μM and 0.3 μM also did not differ in their effects on budbreak percentages. Some explants elongated (Table 27) as in the first collections.

Table 25. Bud breaking in nodal explants of root suckers collected in October, 1991 from two rootstock genotypes.

	Bud break (%) [a]		
	Genotype 1	Genotype 2	Averages
Treatment 1 (plus 0.1 μM GA)	14/19 (74)	19/25(76)	75% [ns]
Treatment 2 (plus 0.3 μM GA)	13/17 (76)	12/12(100)	88% [ns]
Averages	75% [ns]	88% [ns]	

a) Numerator indicates number of budbreaking explants, denominator indicates number of remaining aseptic explants. Values in parentheses are percentages.
ns=not significant at $\alpha_{0.05}$ (Table 26)

Table 26. ANOVA of budbreak frequency in nodal explants from root suckers collected from two rootstock genotypes (October 1991 collections).

Source	SS	df	MS	F-ratio
GA_3 levels	0.306	1	0.306	1.590[ns]
Genotype	0.285	1	0.285	1.737[ns]
GA_3 levels x genotype	0.192	1	0.192	1.170[ns]
Error	11.303	69	0.164	

[ns] = not significant

Table 27. Elongation in nodal explants of root suckers collected in October from two rootstock genotypes.

	Bud elongation(%) *		
	Genotype 1	Genotype 2	Average
Treatment 1(plus 0.1 μM GA)	13/14 (93)	16/19(84)	88%
Treatment 2 (plus 0.3 μM GA)	12/13 (92)	11/12(92)	92%
Averages	92%	88%	

*) Numerator indicates number of elongating explants, denominator indicates number of budbreaking explants. Values in parentheses are percentages.

Two-month growth in length of regenerated shoots showed that the average shoot length did not vary widely among the three treatments tested for each of the rootstock genotypes (Table 28) in both August and October collections from the same trees.

Table 28. Shoot length of 2 month-old regenerated shoots from root sucker nodal explants. Treatment (Tmt.) 1= plus 0.1 μM Ga, Tmt. 2= plus 0.3 μM Ga, Tmt. 3= plus 1.5 μM Ga.

	Average shoot length (cm)*		
	Tmt. 1	Tmt. 2	Tmt. 3
August collections			
Rootstock genotype 1	2.74 (7)	2.70 (4)	2.93 (3)
Rootstock genotype 2	2.97 (4)	2.86 (6)	3.35 (4)
Rootstock genotype 3	3.55 (4)	3.27 (4)	3.26 (5)
October collections			
Rootstock genotype 1	2.67 (8)	3.42 (5)	-
Rootstock genotype 2	3.65 (10)	2.84 (7)	-

*Values in parentheses refer to number of healthy regenerated shoots measured.

b. Stump sprouts. Nodal explants showed bud breaking at the levels of BAP tested (Tables 29 & 30). Bud break percentages were not different among the BAP levels. The two sources of the stump sprouts were also not different in bud breaking frequencies. Bud elongation was observed in some buds that started bursting (Table 31).

Table 29. Bud breaking in nodal explants of stump sprouts grown in various levels of BAP after two weeks in culture.

BAP levels (μM)	Bud break (%)* Tree stump 1	Tree stump 2	Averages
0.1	13/20 (65)	14/19 (74)	70^{ns}
0.5	18/22 (82)	12/22 (54)	68^{ns}
1.0	12/18 (67)	14/19 (74)	71^{ns}
5.0	10/19 (53)	8/18 (44)	48^{ns}
10.0	7/16 (44)	6/15 (40)	42^{ns}
Averages	62^{ns}	57^{ns}	

*Numerator indicates number of budbreaking explants, denominator indicates number of aseptic explants. Values in parentheses are percentages.
^{ns} = not significant

Table 30. ANOVA of bud break frequency in nodal explants from sprouts of two tree stumps.

Source	SS	df	MS	F-ratio
BAP levels	2.682	4	0.671	3.99^{ns}
genotype	0.067	1	0.067	0.288^{ns}
BAP levels x genotype	0.670	4	0.168	0.724^{ns}
Error	41.228	178	0.232	

^{ns} = not significant

Table 31. Bud elongation in nodal explants from stump sprouts grown in various levels of BAP after three weeks in culture.

	Bud elongation (%)*		
	Tree stump 1	Tree stump 2	
BAP levels (μM)			Average
0.1	13/13 (100)	4/14 (29)	64%
0.5	15/18 (83)	5/12 (42)	62%
1.0	8/12 (67)	5/14 (36)	52%
5.0	8/10 (80)	2/8 (25)	52%
10.0	1/7 (14)	1/5 (20)	17%
Averages	69%	30%	

*Numerator indicates number of elongating explants, denominator indicates number of budbreaking explants. Values in parentheses are in percentages.

Nodal explants from summer crown shoots showed bud breaking at various levels of BAP (Table 32). These bud breaking explants did not show a corresponding proportion of bud elongation. There was a low frequency of bud elongation (Table 33). Most of the non-elongated and elongating buds were lost to contamination.

Table 32. Bud break in nodal explants from summer crown shoots (from Sandhill trees) in various levels of BAP after two weeks in culture.

	Bud break (%)*			
	Tree 1	Tree 2	Tree 3	
BAP levels (μM)				Averages
0.1	18/20 (90)	15/16 (93)	10/20 (50)	78%
0.5	10/16 (62)	10/18 (56)	12/19 (63)	60%
1.0	12/23 (52)	12/18 (67)	15/21(71)	63%
5.0	10/22 (45)	8/22 (36)	8/20 (40)	40%
10.0	2/19 (11)	3/18 (17)	4/18 (22)	17%
Averages	52%	54%	49%	

*Numerator refers to no. of bud breaking explants, denominator refers to no. of aseptic explants remaining after the first scoring of contamination (5 days from start of culture). Initial no. of explants=24. Values in parentheses are percentages.

Table 33. Bud elongation in nodal explants from summer crown shoots (from Sandhill trees) in various levels of BAP after three weeks in culture.

	Bud elongation (%)			
	Tree 1	Tree 2	Tree 3	
BAP levels (μM)				Averages
0.1	4/18 (22)	2/15 (13)	0/10 (0)	12%
0.5	3/10 (30)	2/10 (20)	9/12 (75)	42%
1.0	2/12 (17)	1/12 (8)	0/15 (0)	8%
5.0	2/10 (20)	2/8 (25)	3/8 (38)	28%
10.0	0/2 (0)	1/3 (33)	1/4 (25)	19%
Averages	18%	20%	28%	

* Numerator refers to no. of elongating buds, denominator refers to no. of bud breaking explants. Values in parentheses are percentages.

DISCUSSION

The first visible response *in vitro* occurred within 3-4 days as enlargement of the cotyledonary disk and hypocotyl explants. The enlargement was attributed to increases in cell volume (Rogozinska, 1967). Cotyledon and hypocotyl are physiologically immature tissues with a great plasticity (Durand-Cresswell *et al.*, 1985). Food reserves in the form of starch and other molecules in cotyledons are mobilized during tissue culture (Thompson and Thorpe, 1987). Shoot formation was significantly different among the families tested. Root formation was significantly different between Family 1 (0.4%) and Family 3 (7.0%). The *in vitro* expression of organogenesis is a reflection of the intrinsic genetic constitution of a taxon (Tomar and Gupta, 1988); the difference in the morphogenic response between the families may reflect the physiological and genetic heterogeneity of the seed lots in expression of shoot and root formation.

The responsiveness to culture conditions was shown by the high frequencies of callus formation along the cut surfaces of the explants. Callus formed on all the cotyledon and hypocotyl explants. The most pronounced responses were related to BAP levels. The greater the BAP concentration, the higher the frequency of shoot bud formation within the range tested. Shoot bud formation ranged from 0.5% to 48% in 5 and 30 μM BAP. None of these initial treatments, however, stimulated shoot elongation. Shoot elongation was promoted when the cultures were transferred to a lower BAP level (1μM). This stimulation of shoot elongation with a reduction of exogenous cytokinin concentration is similar to that described for soybean (Cheng *et al.*, 1980) and blacklocust (Han, 1991). The test showed that shoot bud formation

frequency in cotyledons and hypocotyls was not significantly different, which suggests that the endogenous cytokinins and their concentration in these explants, plus the added cytokinins in the media constituted an amount favorable to shoot formation. The appearance of roots in medium with a low auxin/cytokinin ratio suggests that some cells of the cotyledons may be producing high levels of auxins, with the endogenous concentration affecting the critical level that triggers root or shoot initiation.

Using seedlings grown indoors as sources of explants gave an advantage of low occurrence of contamination. Nodal explants from these 8 and 10 month-old seedlings regenerated shoots. The favorable effect (higher frequency of bud elongation) of BAP when added alone compared with its effect when combined in the medium with GA or zeatin is similar to that found in chestnut (Vieitez and Vieitez, 1980) in the important role played by BAP in developing axillary shoots in the initial culture. Moreover, the seemingly unfavorable effect of GA in the honeylocust cultures is similar to the adverse effect (halted the development of the cultures) found in chestnut when the growth regulator was added to the basal medium with or without any of the other cytokinins. Whereas zeatin caused a small increase in percent sprouting in chestnuts (Vieitez and Vieitez, 1980), it had no such effect in this study.

In nodal cultures from 10 month-old seedlings, the explants from the basal positions nearer the root were the least responsive. Nodes higher in position (6th to 16th) showed a higher shoot elongation frequency, indicating a differential response of nodes (buds) along the stem axis and the suitability of nodal explants occupying these higher positions for use in micropropagation. This varied from study of *Corylus*

avellana, which showed that shoot proliferation and rooting of explants taken from different locations along the main shoot of the seedlings were always highest in nodes closer to the root (Diaz-Sala *et al.*, 1990).

In cultures of dormant bud explants, buds with and without bud scales opened; young leaves that appeared light green became evident. Some buds retained this greenness throughout the experiment. Other buds however, turned brown, implying a loss of their green pigments. This may have been caused by secretion of phenolic compounds as has been commonly reported in other studies (George and Sherrington, 1984; Debergh and Read, 1991). The buds, regardless of whether bud scales were intact or not, failed to show elongation with various levels of BAP. This is in direct contrast to the response reported for Japanese pear leaf buds, where the presence of budscales delayed expansion of summer dormant buds and bud scale removal reduced the days to bud expansion by half (Yotsuya, *et al.*, 1984).

Five buds in individual cultures of single buds showed initial enlargement of the shoot meristem when observed under the stereoscope. Neither Zeatin nor BAP promoted meristem growth and eventual elongation. Buds cultured as aggregates also did not elongate, although they maintained a relatively high frequency of greenness. The results from dormant buds may indicate that the type or level of cytokinins and auxins tested may not have been at the proper ratio with the endogenous growth regulators to promote morphogenesis, as has been previously postulated (Skoog and Miller, 1957; Minocha, 1987; Zaerr and Mapes, 1982).

Honeylocust is a host to a number of leaf feeders, borers, scale insects and other damaging agents (Blair, 1990). These various organisms could be vectors of

microorganisms that could increase the amount of contaminants in tissue culture. The average contamination frequencies in bud cultures of ornamental honeylocust trees was 10% in contrast to that of buds from Sandhill trees which was 52.91%. This may be due to a later time of collection of the buds from Sandhill trees, 2-3 three months later than the buds of the ornamental trees. It was found in spruce that as the length of time from budbreak increases, so does the frequency of contamination (Keathley, 1984). In addition, in Sandhill a number of weedy herbs and shrubs exist which play host to other vectors of microorganisms that may also attack honeylocust. These weeds are not co-inhabitants of the ornamental trees in their respective sites in the MSU plantings.

The failure to promote shoot development using dormant buds from crown branches from various genotypes collected in the winter led to attempts to identify other types of explants from mature honeylocust trees that are more responsive to tissue culture and which may be used in clonal propagation of mature trees. Rootstocks of three trees from the MSU campus were found bearing root suckers in 1991. The sprouting of root suckers of these trees was probably in response to the damaging effect of lawn mowers that hit the ramifying roots found above ground. There is evidence that the wounding of plant tissues results in the release of "wound hormones" which stimulate cell division (Wareing and Philips, 1981). Some tree species such as aspens, sweetgum, beech and blacklocust regenerate largely by root suckers following disturbance of forest stands by heavy cutting or fire (Kramer and Kozlowski, 1979). The frequency of budbreak was not significantly different among

the three rootstock genotypes and most explants that broke bud continued to elongate until they were placed in rooting media.

The "leafy" phenotype exhibited by elongated shoots from root sucker explants was not observed in cultures of cotyledons and hypocotyls in the present study. Since the regenerated shoots were thought to have developed adventitiously from callus that was derived from the cotyledon and hypocotyl explants and not from a preformed meristem as is found in dormant buds and stem nodal explants, the regenerating meristem and surrounding leaf primordia may not carry the inhibitory substances that are thought to account for the suppressed emergence of the shoot meristem. This may be the result of the fact that cells in callus tissue lack polarity and positional values which are related to the morphogenetic gradients to which cells or organs are subjected and which determine their development (Carr, 1984).

The buds in the root sucker nodal segments continued to elongate in subcultures in media with the same levels of BAP, zeatin, NAA and GA as in the initial cultures. Some factors in the culture media that might have influenced the promotion of shoot elongation were the reduced nitrate concentration, combination of two cytokinins plus the addition of gibberellic acid. Promotion of growth in tissue culture related to these factors has been reported (Rumary and Thorpe, 1983; Goyal and Arya, 1984; Wochok and Sluis, 1980; Altman and Goren, 1974). GA stimulated the growth of axillary buds but also caused internodal elongation and formation of abnormal leaves in nodal cultures from adult trees (Fossard *et al.*, 1978) and the pale green and spindly shoots of plants in culture (Sangwan *et al.*, 1976). The yellowing of leaves in

regenerated honeylocust shoots could be an effect of GA but this needs further experimentation.

Dormant buds from Sandhill that were grown in 1 μM BAP showed callus development at the basal part of the bud in contact with the medium, and slight development of the leaves was observed but failed to elongate. These results, as well as those obtained from buds from ornamental trees, left various aspects unclear as to the requirements of honeylocust dormant buds for shoot elongation. The culture conditions including the composition of the media and the phytohormones used did not stimulate shoot elongation in dormant buds of crown shoots in any treatment. As exogenous growth regulators are known to increase and/or qualitatively alter the levels of endogenous growth regulators (Douglas, 1985) future studies on testing of various phytohormones and their combination with other nutrient factors and other culture conditions may shed light on what could promote shoot elongation for these dormant buds.

Unlike with winter dormant buds, elongation of buds was obtained with root suckers (83% to 93%) and stump sprout nodal explants (17% to 64%) in various BAP levels. A lower average percentage of elongation (range of 6% to 41%) was obtained in nodal explants from summer crown shoots from three trees 14 years of age grown in various BAP levels. The capacity for clonal propagation is closely linked to the genetic and physiological factors that control the transition from juvenile to mature growth, and phase change or maturation in woody plants results in increased difficulty in vegetatively propagated tree species (Bonga, 1982; 1987; Greenwood, 1987). Some studies linked the presence/absence of some compounds in tissues such as

hydroxycinnamic acid amides (Cabanne *et al.*, 1981) and peroxidase (Moncousin, 1982 mentioned by Nozeran in 1984) with the juvenile or mature state of the plant. To overcome the problem in tree species, methods such as cytokinin application, serial culture of explants, and grafting have been employed to rejuvenate mature tissues for propagation (Greenwood, 1987; Franclet, 1983).

The results on honeylocust are comparable with those found on other trees in that the growth response of crown shoots diminished with age (reviewed by Franclet, 1983; Hackett, 1987). On the other hand, aspen, cherry trees, *Eucalyptus* and *Sequoia* and *Quercus* were rapidly propagated *in vitro* by basal sprouts (Chalupa, 1983; reviews by Franclet, 1983). Postulates about aging in tree species were reviewed by Meier-Dinkel and Kleinschmit (1990). Among them was that ontogenetic aging seems to be located in the terminal meristem and is carried onto its lateral meristem when they are formed (Robinson and Wareing, 1969). Based on this, basal parts of a tree are considered juvenile whereas the top is ontogenetically the most mature. It has also been supposed that with increasing size of a tree, the shoot apex becomes more remote from the roots and the level of endogenous gibberellin in the apex declines (Meier-Dinkel and Kleinschmit, 1990).

Finally, this study showed that the use of root suckers and stump sprouts as sources of explants for mature tree micropropagation of honeylocust is more feasible than the use of dormant buds and crown shoot nodal explants. It appeared that budbreak in nodal explants of summer crown shoots could be stimulated by the BAP concentrations tested in this study, but the same plant growth regulator and the concentrations tested stimulated a low frequency of bud elongation. Elongation of

buds

used

MS

of h

tree

gra

high

wou

stim

buds from nodal explants from root suckers was obtained from rootstocks that were used in the budding of desirable cultivars of ornamental honeylocust planted on the MSU campus. An important application of these results would be in the propagation of honeylocust trees with desirable qualities for agroforestry or for ornamental use. In trees where the rootstock and the crown have the same genotype, i.e. the tree is not grafted, using their sprouts as explants is expected to yield a culture system with higher efficiency for propagating these elite genotypes. The use of these techniques would be increased by application of cultural practices, such as wounding, that stimulate the development of root or trunk sprouts.

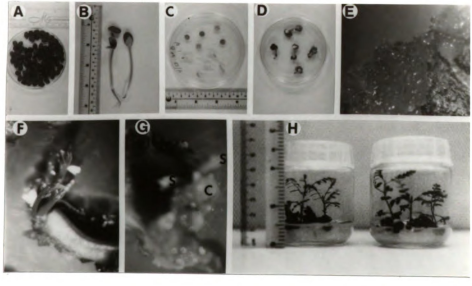

Figure 1. Tissue culture of cotyledon and hypocotyl segments from 5 day-old germinating honeylocust seedlings. **(A)** Honeylocust seeds. **(B)** Five day-old seedlings. **(C)** Cotyledon disks and hypocotyl segments as explants. **(D)** Callus formed in hypocotyl segments. **(E)** Callus formed in cotyledon disk. **(F)** Shoots regenerated from hypocotyl explants. **(G)** Shoots regenerated from cotyledon explant. **(H)** Shoot elongation in 1 μM BAP.

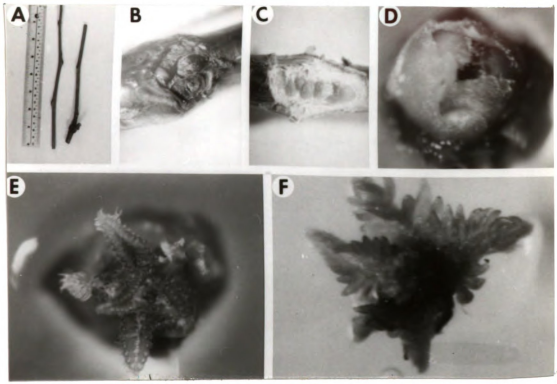

Figure 2. Tissue culture of honeylocust dormant buds from crown shoots. **(A)** Stem segments bearing winter dormant buds. **(B)** Dormant buds in a single node. Dissected node showing five dormant buds. **(D)** Single bud with overlapping bud scales. **(E)** Two week-old culture of single buds showing unfolded leaves. **(F)** Non-elongating bud in 6 month-old culture.

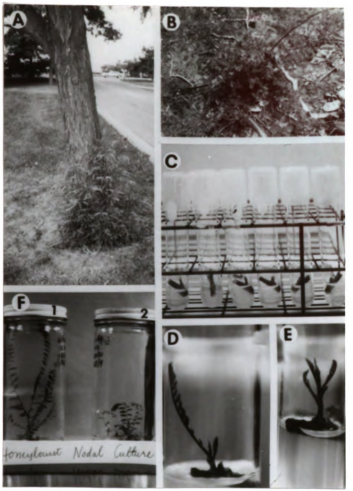

Figure 3. Tissue culture of explants from root suckers and stump sprouts. **(A)** Root suckers of an ornamental honeylocust tree. **(B)** Stump sprouts of honeylocust. **(C)** Nodal segment explants. **(D), (E) & (F_1)** Elongated shoots from root sucker nodal explants. **(F_2)** "Leafy" phenotype.

LITERATURE CITED

Aitken-Christie, J. 1984. Micropropagation of *Pinus radiata*. The Plant Propagator 30(3):9-11.

Allen, O.N. and E. K. Allen. 1981. The Leguminosae. The University of Wisconsin Press, Wisconsin, USA. pp. XI-XXIII, 298-300.

Altman, A. and R. Goren. 1974. Growth and dormancy cycles in *Citrus* bud cultures and their hormonal control. Physiol. Plant. 30: 240-245.

Barnes, B. V. and W. H. Wagner. 1981. Michigan Trees. The University of Michigan Press, Ann Arbor. p.166.

Basbaa, A., A. Geslot and P. Neville. 1993. Multiplication végétative in vitro de *Gleditsia triacanthos* L. Rev. Cytol. Biol. végét.-Bot.16, 147-161.

Blair, R. 1990. *Gleditsia triacanthos* L. In: Silvics of North America. V. 2. Hardwoods, R. M. Burns and B. Honkala, (Tech. Coordinators). Forest Service, United States Department of Agriculture. pp. 358-364.

Bonga, J.M. 1987. Clonal propagation of mature trees: Problems and possible solutions. In: Cell and Tissue Culture in Forestry, J.M. Bonga and D. J. Durzan (eds.), Martinus Nijhoff Publishers. Dordrecht/Boston/Lancaster. pp. 249-271.

Bonga, J.M. 1982. Vegetative propagation in relation to juvenility, maturity, and rejuvenation. In: Tissue Culture in Forestry, J.M. Bonga and D.J. Durzan (eds.), Martinus Nijhoff Publishers. Dordrecht. pp. 387-412.

Cabanne, F., M.A. Dalebroux, J. Martin-Tanguy and C. Martin. 1981. Hydroxycinnamic acid amides and ripening to flower of *Nicotiana tabacum* var. xanthi n.c. Physiol. Plant. 53:399-404.

Carr, D.J. 1984. Positional information and plant morphology. In: Positional Controls in Plant Development, P.W. Barlow and D.J. Carr (eds.). Cambridge University Press, London. pp. 349-372.

Chalupa, V. 1983. Micropropagation of conifer and broadleaved forest trees. Communicationes Instituti Forestalis Cechosloveniae, Vol. 13, pp. 7-39.

Chase, S.B. 1947. Propagation of thornless honeylocust. J. Forestry 45: 715-722.

Cheng, T., H. Saka, and T.H. Voqui-Dinh. 1980. Plant regeneration from soybean cotyledonary node. Plant Sci. Lett. 19:91-99.

Conover, W. J. 1971. Practical nonparametric statistics. John Wiley and Sons Inc., New York. pp. 256-263.

Debergh, P.C. and P.E. Read. 1991. Micropropagation. In: Micropropagation *Technology and Application*, P.C. Debergh and R.H. Zimmerman, (eds.), Kluwer Academic Publishers. Dordrecht. pp. 1-13.

Diaz-Sala, C., C. Rey and R. Rodriguez. 1990. Recovery of transient juvenile capacities during micropropagation of filbert. In: Plant Aging: Basic and Applied Approaches, R. Rodriquez, R. Sanchez Tames and D.J. Durzan, (eds.). Plenum Press, New York. pp. 27-36.

Douglas, G. C. 1985. Formation of adventitious buds in stem internodes of *Populus* hybrid TT32 cultured *in vitro*: Effects of sucrose, zeatin, IAA and ABA. J. Plant Physiol. Vol. 121. pp. 225-23.

Dunstan, D.I. and T.A. Thorpe. 1986. Regeneration in Forest Trees. In: Cell Culture and Somatic Cell Genetics of Plants. V. 3. Academic Press, Inc., pp. 223-241.

Durand-Cresswell, R., M. Boulay and A. Franclet. 1985. Vegetative propagation of *Eucalyptus*. In: Tissue Culture in Forestry, J.M. Bonga and D.J. Durzan, (eds.), Martinus Nijhoff/Dr W. Junk Publishers, Dordrecht. pp. 150-181.

Fossard, R.A. de, M.T. Bennett, J.R. Gorst and R.A. Bourne. 1978. Tissue culture propagation of *Eucalyptus ficifolia* F. Muell. The International Plant Propagators' Society 28:427-434.

Franclet, A. 1983. Rejuvenation: Theory and practical experiences in clonal silviculture. Nineteenth Canadian Tree Improvement Association Meeting Proceedings, August 22-26, Toronto, Canada. pp. 96-134.

George, E.F. and P.D. Sherrington. 1984. Plant Propagation and Tissue Culture. Exegetics Ltd. Edington, Westbury, England. 709 p.

Gold, M.A. 1984. Honeylocust (*Gleditsia triacanthos* L.): Genetic variation and potential use as an agroforestry species. Ph.D. Thesis. Michigan State University, East Lansing, Michigan, 163 p.

Gold, M.A. and J.W. Hanover. 1993. Honeylocust (*Gleditsia triacanthos*), A multipurpose tree for the temperate zone. The International Tree Crops Journal, 7:189-207.

Goyal, Y. and H. Arya. 1984. Tissue culture of desert trees: I. Clonal multiplication of *Prosopis cineraria* by bud culture. J. Plant Physiol. 115:183-189.

Greenwood, M.S. 1987. Rejuvenation of forest trees. Plant Growth Regulation 6:1-12. Martinus Nijhoff Publishers, Dordrecht, Netherlands.

Hackett, W.P. 1987. Juvenilty and maturity. In: Cell and Tissue Culture in Forestry, J.M. Bonga and D. J. Durzan (eds.), V. 1. Martinus Nijhoff Publishers, Boston. pp. 216-231.

Han, Kyung-Hwan. 1991. *Agrobacterium rhizogenes*-mediated transformation and plant regeneration from normal and transgenic tissues of *Robinia pseudoacacia* L. Michigan, 96 p.

Heit, C.E. 1967. Part 6: Hardseededness - a critical factor. Amer. Nurseryman. 1125:10-12, 88-96.

Hollander, M. and D. Wolfe. 1972. Nonparametric statistical methods. John Wiley and Sons, New York. pp. 114 -129.

Keathley, D.E. 1984. Micropropagation of mature spruce. Proceedings of the International Symposium in Recent Advances in Forest Biotechnology. Traverse City, Michigan. pp. 58-63.

Kramer, P.J. and T.T. Kozlowski. 1979. Physiology of Woody Plants. Academic Press, New York. pp. 61, 150-152.

Meir-Dinkel, A. and J. Kleinschmit. 1990. Aging in tree species: present knowledge. In: Plant Aging- Basic and Applied Approaches. R. Rodriguez, R. Sanchez Tames and D.J. Durzan, (eds.), Plenum Press, New York. pp.191-197.

Minocha, S. 1987. Plant growth regulators and morphogenesis in cell and tissue culture of forest trees. In: Cell and Tissue Culture in Forestry, V.1, J.M. Bonga and D. J. Durzan, (eds.)., Martinus Nijhoff Publishers. Dordrecht/Boston/Lancaster. pp. 50-66.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum 115: 473-497.

Nozeran, R. 1984. Integration of organismal development. In: Positional Controls in Plant Development, P.W. Barlow and D.J. Carr (eds.). Cambridge University Press, Cambridge. pp.375-401.

Robertson, K.R. and Y.T. Lee. 1976. The genera of Caesalpinioideae (Leguminosae) in the southeastern United States. J. Arn. Arb. 57(1): 1-53.

Robinson, L. W. and P. F. Wareing. 1969. Experiments on the juvenile-adult phase change in some woody species. New Phytol. 68: 67-78

Rogozinska, J. H. 1967. Triacanthine, growth substances and the *in vitro* culture of *Gleditsia triacanthos* L. Bull. de L'Academie Polonaise des Sciences Cl.II.- Vol. XV, No. pp. 313-317.

Rogozinska, J. H. 1968. The influence of growth substances on the organogenesis of Honey locust shoots. (In Polish). Acta. Soc. Bot. Pol., 37:485.

Rogozinska, J. H. 1969. Organogenetic differentiation in honey locust cotyledons. Bull. de L'Academie Polonaise des Sciences Cl.II. Vol. XVII, No. 11-12, pp. 721-725.

Rumary, C. and T. A. Thorpe. 1983. Plantlet formation in black and white spruce. I. *In vitro* techniques. Can. J. For. Res. 14:10-16.

Sanchez, M.C. and A. M. Vietez. 1991. *In vitro* morphogenetic competence of basal sprouts and crown branches of mature chestnuts. Tree Physiology 8:59-70.

Sangwan, R. S., B. Norreel and H. Harada. 1976. Effects of kinetin and gibberellin A_3 on callus growth and organ formation in *Limnophila chinensis* tissue culture. Biol. Plant. 18:126-131.

Skoog, F. and C.O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp. Soc. Exp. Biol. 11:118-30.

Smith, J. R. 1950. Tree crops. A Permanent Agriculture. The Devin-Adair Company. New York, pp. 65-79.

Steel, R. and J. Torrie. 1980. Principles and procedures of statistics. McGraw-Hill Book Co., New York. p. 236.

Thompson, M.R. and T.A. Thorpe. 1987. Metabolic and non-metabolic roles of carbohydrates. In: Cell and Tissue Culture in Forestry. V. 1, J.M. Bonga and D. J. Durzan, (eds.), Martinus Nijhoff Publishers. Dordrecht/Boston/ Lancaster. pp. 89-112.

Thorpe, T.A. 1990. Organogenesis: Structural, physiological, and biochemical aspects. In: Plant Aging- Basic and Applied Approaches. R. Rodriguez, R.

Sanchez Tames and D.J. Durzan, (eds.), Plenum Press, New York. pp.191-197.

Thorpe, T. A., I. S. Harry and P. P. Kumar. 1991. Application of micropropagation to forestry. In: P.C. Debergh and R.H. Zimmerman, (eds.), Micropropagation *Technology and Application* Kluwer Academic Publishers. Dordrecht. pp. 311-336.

Tomar, U. and S. Gupta. 1988. *In vitro* plant regeneration of leguminous trees (*Albizia* spp.). Plant Cell Rep. 7: 385-388.

Vieitez, A. M. and L. Vieitez. 1980. Culture of chestnut shoots from buds *in vitro*. J. Hort. Sci. 55 (1):83-84.

Vieitez, A.M. and M.L. Vieitez. 1982/83. *Castanea sativa* plantlets proliferated from axillary buds cultivated *in vitro*. Scientia Hort. 18:343-351.

Wareing, P.F. and I.D.J. Philips. 1981. Growth and Differentiation in Plants. Pergamon Press. New York. pp. 154-167.

Wilkinson, L. M. Hill, J. Welna and G. Birkenbeuel. 1992. Systat Statistics. Systat Inc., Il., 750 p.

Wochok, Z. and C. Sluis. 1980. Gibberellic acid promotes *Atriplex* shoot multiplication and elongation. Plant Science Letters 17: 363-369.

Yotsuya, T., I. Takao, M. Sawano, T. Nakanishi and T. Ozaki. 1984. Effects of bud scales and gibberellins on dormancy of *in vitro* cultured Japanese pear leaf buds. Scientia Hortic. 24:177-184.

Zaerr, J.B. and M.O. Mapes. 1982. Action of growth regulators. In: Tissue Culture in Forestry, J.M. Bonga and D.J. Durzan (eds.), Martinus Nijhoff/Dr W. Junk Publishers. Boston. pp. 231-255.

CHAPTER 2

Rooting and acclimatization of plantlets in tissue culture of *Gleditsia triacanthos* L. (honeylocust)

ABSTRACT

Initial attempts to induce rooting failed in micropropagated shoots derived from cultures of cotyledon, hypocotyl segments and buds from nodal segments of root suckers cultured in one-tenth strength of MS basal medium without sucrose and supplemented with IBA or NAA in continuous exposure to alternating light/dark regime.

Rooting of micropropagated shoots (derived from cotyledon nodes and hypocotyl segments and buds from nodal segments of 10 month-old seedlings, root suckers and stump sprouts of mature trees) was obtained from cultures in MS medium containing nitrates at half strength, 10 g/l sucrose and supplemented with IBA or NAA or a mixture of both auxins. Cultures were initially incubated in the dark for a 9-day period and then subcultured in an auxin-free modified MS medium in an alternating light/dark regime.

The rooted shoots successfully acclimatized in a planting medium consisting of peat/perlite/vermiculite mixture in a humid and constantly lighted environmental condition.

INTRODUCTION

Rooting is of practical importance to the propagation and multiplication of plants. Root formation is influenced by various interacting exogenous and endogenous factors, such as genotype, ontogenetic age and environmental factors such as light, temperature, aeration and mineral nutrition (Mullins, 1985; Nemeth, 1986; Nanda *et al.*, 1973). These factors are thought to exert their various effects through the medium of endogenous growth substances which in turn direct the differentiation and emergence of adventitious roots (Mullins, 1985).

Applied nutrients may not generally be needed during rooting because endogenous nutrients are basipetally transported from the shoot, except possibly boron, which is related to membrane permeability and sugar transport, and nitrogen, which is found in proteins and nucleic acids (Eliasson, 1978; Gaspar and Coumans, 1987). Some studies, however, show that control of adventitious root initiation and growth is subject to several interacting factors, such as physiological age, and both hormonal and nutrient status of the explant (Thompson and Thorpe, 1987). Studies showed that synthetic IAA as well as NAA and IBA were active in adventitious root formation (Skoog and Miller, 1957; Smith and Thorpe, 1975). Skoog and Miller (1957) showed that the direction of differentiation of tobacco callus was determined by the ratio of exogenous cytokinin and auxin: a low level of cytokinin in relation to applied auxin favored the differentiation of roots. The determination of shoot/root formation is generally dependent on the cytokinin/auxin ratio, however, the critical balance of growth regulation is in the tissue itself at the organ-forming loci (Nemeth, 1986). This seems to imply the importance of the endogenous levels of growth regulators

relative to the level of exogenous auxins applied. The effects of exogenous regulators are related to the histologic events in adventitious root initiation (Smith and Thorpe, 1975; Haissig, 1972; Nanda *et al.*, 1973; Kato *et al.*, 1978).

Auxins are the main factors involved in root formation (Nemeth, 1986) and studies on rooting *in vivo* showed that these growth substances act to regulate root growth in both stimulatory and inhibitory capacities (Scott, 1972; Torrey, 1965; Nemeth, 1986). At extremely low auxin concentration, roots are stimulated in their elongation and at progressively higher auxin concentrations are inhibited (Torrey, 1965). If the shoots are vigorous, enough natural auxins may be produced to give good rooting in hormone-free medium but added auxins usually stimulate root initiation (Wetherell, 1982).

Adventitious root formation can be divided into two developmental stages - initiation of primordia following cutting or wounding and root emergence (James, 1983). The supply of auxins in the first stage is critical while it is not required or even inhibitory in the second stage (James,1983; Smith and Thorpe, 1975; Chalupa, 1983; Vieitez and Vieitez, 1982). Auxins influence the induction of meristematic activity in the root (Scott, 1972). Naphthaleneacetic acid (NAA) and indolebutyric acid (IBA), which are synthetic analogues of the naturally-occurring auxin indoleacetic acid (IAA), are routinely used in plant tissue culture. They are suspected to have longer half-life, they stimulate less ethylene production than IAA, and plants possess mechanisms that reduce the effectiveness of IAA by conjugating it with other compounds (Schwarz, 1987; Blazich, 1988). Mixtures of certain root-inducing substances were found more effective for root formation in cuttings than equivalent

concentrations of the individual substances (Hitchcock and Zimmerman, 1940). It is in the first developmental stage of root formation that auxins are critical during cell division leading to the formation of root primordia. Root emergence and root elongation stages do not require auxins (James, 1983; Davis and Keathley, 1987; Nemeth, 1986; Gupta *et al.*, 1980; Schwarz, 1987; Haissig, 1972).

A dark regime or etiolation of basal ends of materials to be rooted has been reported to be favorable to root formation (Druart *et al.*, 1982; Kawase, 1965; Hammerschlag, 1982; reviews by Nemeth, 1986), although several studies on micropropagation had shown rooting without this treatment (Davis and Keathley, 1987; Han, 1991; Gupta *et al.*, 1980; Chalupa, 1983).

After shoots and roots are developed the plantlets are transferred from tissue culture tubes to the planting medium. Acclimatization is an essential and critical stage of the micropropagation process, since the plantlets which have been adapted to the highly humid environment inside the culture tubes must be readapted to a less humid environment. The final measure of success of micropropagation depends on the ability to transfer plants out of the culture tubes with high percentage of success.

Tissue-cultured plants are difficult to transplant for two main reasons- their heterotrophic mode of nutrition, although plantlets may appear "fully functional" physiologically, and their poor control of water loss which is attributable to slow stomatal response (Conner and Thomas, 1981; Brainerd and Fuchigami, 1981). The importance of environmental conditions during transplanting such as the growing environment, planting medium, fertilization and disease has been reviewed elsewhere

(Conner and Thomas, 1981; Hutchinson and Zimmerman, 1987; Dunstan and Turner, 1984).

The objectives of this study were to induce rooting in shoots regenerated from juvenile and mature honeylocust explant sources (given in Chapter I) and to acclimatize such plantlets.

MATERIALS AND METHODS

A. Rooting

1. Experiment 1

a. Plant materials. Regenerated shoots that attained at least 2 cm in height from tissue cultures of cotyledon, hypocotyl and nodal segments of root suckers were tested for rooting. Some shoots were excised directly from the primary explants while others used were excised previously from the primary explants and just trimmed.

b. Culture media and environmental conditions. Excised shoots from hypocotyls and cotyledons were individually cultured in test tubes (18 x 150 mm) each containing 10 ml of medium. Shoots from root suckers were cultured individually in glass jars (5 x 13 cm) each containing 35 ml of medium. The medium was 1/10 strength of Murashige and Skoog (MS) basal medium without sucrose (Davis and Keathley, 1987; Han, 1991). It was supplemented with auxins: 0.5 μM IBA, 1.0 μM IBA, 0.5 μM NAA and 1.0 μM NAA, solidified with 0.8% agar and adjusted to pH 5.8. The MS control medium did not contain auxin. Ten shoots from cotyledon and hypocotyl explants and 15 shoots from root suckers were cultured per treatment. Cultures were placed in a growth chamber at 26-27°C with an 18 hr. light/6 hr. dark lighting regime at 30-45 μMol/s/m^2/PAR using a cool white fluorescent lamp (F96T12/CW/VHO).

2. Experiment 2

a. Plant materials. Shoots were obtained from tissue cultures of cotyledon nodes and hypocotyl segments in 15 or 30 μM BAP. Developing shoots were transferred to 1 μM BAP and shoots attaining at least 2 cm in height were used for rooting as in Experiment 1.

b. Culture media. The root induction medium consisted of modified MS basal medium (½ strength NH_4NO_3 and KNO_3; Vieitez and Vieitez, 1982) augmented with 10 g/l sucrose. The medium was solidified with agar as in Experiment 1. The auxin concentrations were 1 mg/l NAA, 2 mg/l NAA, 1 mg/l IBA, 2 mg/l IBA, and a mixture of 1 mg/l IBA and 1 mg/l NAA. BAP at 0.02 μM concentration was added to each treatment. A medium without auxin and cytokinin served as control. Twenty-four shoots were cultured per treatment. The cultures were kept dark for nine days and placed in the growth chamber in conditions as in Experiment 1. After the dark period, the shoots were subcultured to an auxin-free modified MS medium under 16 hr. light/8 hr. dark lighting regime in the growth chamber.

c. Characters scored and analysis of data. Fifteen to twenty days from the start of rooting, the plantlets and the agar were removed from the tubes. Data were taken on percent rooted shoots, number of roots and root length.

3. Experiment 3

a. Plant materials. Shoots, at least 2 cm long that regenerated from nodal segments of root suckers (Figure 1A) and stump sprouts were freshly trimmed. Ten for one genotype, fifteen shoots for the second genotype, and 20 shoots from stump sprouts of one tree were cultured per treatment (treatment size was based on explant availability). Shoots that originated from the different nodal positions of 10 month-old seedlings were excised from the original explant and also placed in root induction medium. Nine shoots (eighth position), 15 shoots (tenth position), 15 shoots (twelfth position), 9 shoots (fourteenth position) and 5 shoots (sixteenth position) were placed in root induction medium.

b. Culture media and conditions. Two treatments showing the highest rooting in Experiment 2 were used for root suckers and stump sprouts. These were: (1) 2 mg/l NAA and (2) a mixture of 1 mg/l NAA and 1 mg/l IBA. Only modified MS medium plus 2 mg/l NAA was used for shoots from nodes of 10 month-old seedlings due to small number of samples from some node positions. BAP was added to all the treatments as in Experiment 2.

c. Characters scored and analysis of data. Data taken were as in Experiment 2. An analysis of variance for factorial experiment was performed (2 rootstock genotypes and 2 combinations and levels of NAA and IBA) on total root length. Statistical analysis was not performed on rooting data on shoots from different node positions due to small sample size of explants from some node positions.

B. Acclimatization

The rooted shoots were planted in waxed cardboard pots (10 cm x 10 cm) which contained a peat:perlite:vermiculite (1:1:1) mixture. The planting medium was watered and allowed to soak overnight before planting. Twenty-six rooted shoots derived from cotyledon and hypocotyl, eighteen rooted shoots from root suckers and nine rooted shoots from stump sprouts were tested for acclimatization. The plantlets in the paper pots were covered with two layers of white transparent plastic wrap for 14 days. Light intensity in the growth stand was 70-80 μMol./s/m^2/PAR. Watering was 3x weekly and fertilization 2x monthly with 0.5 g/l of 20-20-20 (NPK) fertilizer to give 100 ppm of nitrogen. Percentage survival was taken after one month and plant height measured after eight months.

RESULTS AND DISCUSSION

Rooting

No roots were formed on shoots used in Experiment 1 in which the medium consisted of 1/10 strength MS basal medium supplemented with auxins, without sucrose, and cultures given an alternating 16 hr. light/8 hr. dark regime.

In Experiment 2, the earliest time observed for root emergence was seven days from the initiation of exposure of cultures to alternating 16 hr. light/ 8 hr. dark period. No roots were formed in the control and in the medium with 1 mg/l IBA (Table 1). Less than 50% rooting was obtained in the other four treatments; rooting frequency was highest (42%) in MS medium with 2 mg/l NAA. Rooted shoots are shown in Figure 2A & B. Callus often formed at the base of shoots which were rooting. The range in root number and mean total root length are also shown in Table 1.

In Experiment 3, in both rootstock genotypes and treatments more than 50% root formation occurred (Table 2). Total root length was not significantly different between the two rootstock genotypes and between the two treatments (2 mg/l NAA and 1 mg/l each of NAA and IBA) tested (Tables 2 & 3). Rooted shoots are shown in Figure 1C.

A similar response was found in stump sprouts; mean total root length was not significantly different between the two treatments (Table 4).

Table 1. Root formation in honeylocust shoots from cotyledons and hypocotyl explants.

Treatment			Rooting*	Root number range	Mean total root length (cm)
	Auxin level (mg/l)				
No.	NAA	IBA			
1	1	0	5/24(21)	0-4	4.2
2	0	1	0/24(0)	0	-
3	2	0	10/24(42)	0-5	9.1
4	0	2	8/24(33)	0-3	4.0
5	1	1	9/24(38)	0-4	6.0
6	0	0	0/24(0)	0	-

*numerator refers to no. of rooting shoots, denominator refers to initial no. of shoots placed in rooting medium, values in parentheses refer to percentage of rooting.

Table 2. Rooting of shoots regenerated from root suckers of two genotypes in MS medium containing 2 mg/l NAA (tmt.1) and 1 mg/l each of NAA and IBA. Scores were taken 3 weeks from start of appearance of roots.

	Root formation (%)[1]			Mean total root length (cm)		
Rootstock	Tmt.1	Tmt.2	Average	Tmt.1	Tmt. 2	Average[2]
1	6/10(60)	8/10(80)	70%	2.9	3.9	3.4 ^{ns}
2	8/15(53)	11/15(73)	63%	4.6	4.7	4.6 ^{ns}
	Average[3]: Tmt. 1= 56^{ns}	Tmt. 2= 76^{ns}		Average[3]: Tmt. 1=3.8^{ns}	Tmt. 2=4.3^{ns}	

[1] numerator indicates no. of rooting shoots, denominator indicates initial no. of shoots placed in rooting medium. Values in parentheses are percentages.
[2] & [3] ns=not significant at $\alpha_{0.05}$ (Table 3).

Table 3. ANOVA of total root length of shoots regenerated from root suckers of two genotypes in medium containing 2 mg/l NAA (tmt.1) and 1 mg/l each of NAA and IBA.

Source	SS	df	MS	F-ratio
Genotype	10.470	1	10.470	2.155^{ns}
Treatment	3.105	1	3.105	3.996^{ns}
Genotype x treatment	0.777	1	0.777	0.160^{ns}
Error	140.909	29	4.859	

Table 4. Root formation in shoots regenerated from stump sprouts in medium containing 2 mg/l NAA (tmt.1) and 1 mg/l NAA plus 1 mg/l IBA (tmt. 2). Scores were taken three weeks from the start of appearance of roots.

	Root formation[1)]	Mean total root length (cm)[2]
Tmt. 1	11/20(55)	2.83[ns]
Tmt. 2	6/20(30)	3.18[ns]

[1)] numerator refers to no. of rooting shoots, denominator refers to initial no. of shoots for root induction, values in parentheses refer to percentage rooting.
[2)] T-test (t= -0.151, p-value = 0.883)
ns= not significant at $\alpha_{0.05}$

Shoots that regenerated from nodes in all positions formed roots (Table 5) varying from 0 to 6 roots per shoot and 2.5 cm to 4.3 cm in mean total root length.

Table 5. Root formation of honeylocust shoots regenerated from different nodal segments of 10 month-old seedlings cultured in MS medium plus 2 mg/l NAA.

Node position	Rooting [1)]	Range in root no.	Mean total root length (cm)
8	5/9(56)	0-4	3.80
10	11/15(73)	0-5	3.06
12	6/15(40)	0-6	4.38
14	5/9(56)	0-3	3.40
16	4/5(80)	0-3	2.56

[1)] numerator indicates no. of rooting shoots, denominator indicates initial no. of shoots placed in rooting medium; values in parentheses are percentages.

Acclimatization

The survival performance of the plantlets transferred to planting medium is given in Tables 6 and 7. Eight, four and seven plants previously rooted in three kinds of MS-based media (plus 1 mg/l each of NAA and IBA, 2 mg/l NAA, 2 mg/l IBA, respectively) survived with a mean height of 66 cm. Eleven plants that regenerated from nodal segments of root suckers reached a mean height of 73 cm at 8-month age. Five plants that regenerated from nodal segments of stump sprouts reached a mean height of 36 cm (Table 7). Acclimatized plants are shown in Figure 1D.

Table 6. *In vivo* performance of plantlets derived from cotyledon and hypocotyl explants.

Modified MS-based rooting Medium	No. of transplanted plantlets	No. of surviving plants at three-month time	Mean Height (cm) at 8 month age
1 mg/l NAA plus 1 mg/l IBA	9	8	68.37 ± 17.24
2 mg/l NAA	9	4	66.05 ± 28.05
2 mg/l IBA	8	7	64.10 ± 33.97

Table 7. *In vivo* performance of plantlets derived from root suckers and stump sprouts.

Primary Explant	No. of Plantlets	No. of surviving plantlets at three-month time	Mean height (cm) at 8 months age
Root suckers (Wilson)	9	7	75.00 ± 36.95
Root suckers (Library)	9	4	71.05 ± 29.60
Stump sprouts	9	5	36.20 ± 21.80

The results show that the conditions set in these experiments for root induction were favorable for some honeylocust shoots derived from both juvenile and mature explant sources. MS medium with nitrates at half strength, auxins, dark treatment and the auxin-free modified MS medium after the dark period are the major factors that are usually mentioned for promotion of rooting of some woody species (reviews in Davis *et al.*, 1988; Nemeth, 1986).

The medium that promoted root initiation in honeylocust contained half strength of the nitrate component of the original MS medium and contained sucrose. This is in line with many reports in the literature. Rooting of micropropagated black locust shoots was obtained in MS medium with one-tenth nitrates (Davis and Keathley, 1987; Han, 1991), in culture from basal shoots of mature chestnuts grown in Gresshoff and Doy's basal medium with one-third macronutrient concentration (Sanchez and Vieitez, 1991), in chestnuts grown in a medium at half strength nitrates (Vieitez and Vieitez, 1982) and in rose, an increase in number and length of roots per shoot by a one-eighth total reduction of the total nitrogen in MS (Hyndman *et al.*, 1982).

Other studies have indicated that many factors, including exogenous and endogenous auxins play crucial roles in the process of rooting (Gaspar and Coumans, 1987; Thompson and Thorpe, 1987). The favorable effect of NAA and IBA in this study on honeylocust is consistent with the results of numerous studies (reviews by Haissig, 1974a & b) although in contrast to findings that IBA was more effective than NAA for most species (Hitchcock and Zimmerman, 1939). In the present study, 1 mg/l IBA resulted in no rooting in shoots derived from hypocotyl and cotyledon explants, yet, 2 mg/l NAA, 2 mg/l IBA and a mixture of 1 mg/l each of these two

auxins were favorable to rooting of shoots from both juvenile- and mature- derived explants. Several other studies had previously shown that mixtures of NAA and IBA, and NAA and IAA were more effective for root formation in cuttings than equivalent concentration of the individual substances (Hitchcock and Zimmerman, 1940; Khosh-Khui and Sink, 1982).

The 9-day continuous dark period during the root induction stage could be another determining factor for the promotion of rooting in the honeylocust shoots, because when shoots were exposed to alternating 18 hr. light/6 hr. dark lighting regime, no roots formed. Similar results have been reported on rooting of woody plants in tissue culture which requires darkness or low irradiance during the auxin-sensitive phase (reviews by Nemeth, 1986). It is interesting to note, however, that several tree species do not require this condition for rooting (Chalupa, 1983; Davis and Keathley, 1987; Han, 1991).

Based on these experiments, micropropagated honeylocust shoots will be expected to form roots in a modified MS medium containing nitrates at half strength, 10 g/l sucrose and supplemented with IBA (2 mg/l) or NAA (1 mg/l or 2 mg/l) or a mixture of 1 mg/l IBA and 1 mg/l NAA grown in a 9-day dark period and subcultured in an auxin-free modified MS media in an alternating 16 hr. light/8 hr. dark lighting regime. In all the root induction experiments using 2 mg/l NAA and 1 mg/l each of NAA and IBA, these two treatments were not significantly different in their effects on percentage rooting and total root length. This implies that the concentration of the plant growth regulators added to the medium may be optimum for root induction. Other hormonal levels and combinations could be tested to refine the results obtained herein.

The rooted shoots can be acclimatized in a planting medium of peat/perlite/vermiculite (1:1:1) mixture initially kept humid by sufficient watering and plastic covering of the entire potted plantlets under constant illumination.

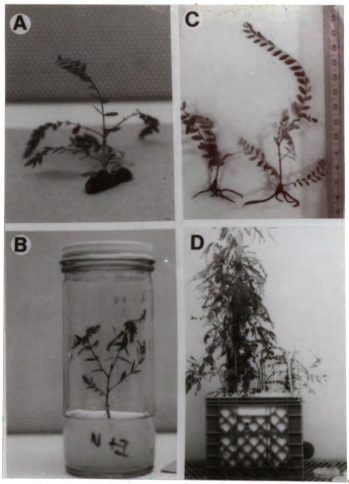

Figure 1. Rooting and acclimatization of plantlets derived from root sucker nodal explants. **(A)** Elongated shoot from bud of root sucker node prepared for transfer to root induction medium. **(B)** Shoots in modified MS medium supplemented with 1 mg/l NAA and 1 μM IBA. **(C)** Rooted shoots after given an initial 9 day-dark pretreatment in modified MS medium supplemented with 1 mg/l each of NAA and IBA and transferred to an auxin-free medium. **(D)** Nine month-old honeylocust plantlets derived from root sucker nodal segments acclimatized in a peat/perlite/vermiculite mixture.

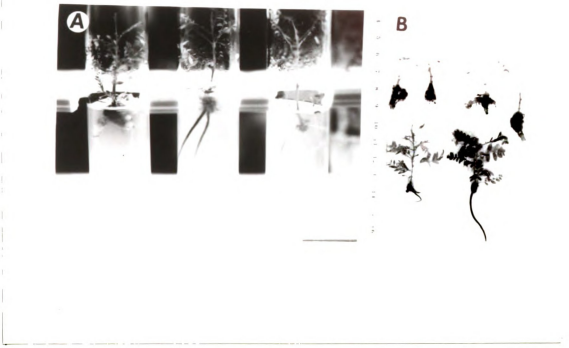

Figure 2. Rooting of shoots derived from cotyledon nodal and hypocotyl segment explants. **(A) & (B)** Rooted shoots were initially given a 9 day-dark treatment in modified medium containing 2 mg/l NAA and transferred to an auxin-free medium.

LITERATURE CITED

Blazich, F. 1988. Chemicals and formulations used to promote adventitious rooting. In: Adventitious root formation in cuttings, Davis, T., B. Hassig and N. Sankhla. (eds.). Advances in Plant Sciences Series, V.2. Dioscorides Press, Oregon. pp. 132-149.

Brainerd, K.E. and L.H. Fuchigami. 1981. Acclimatization of aseptically cultured apple plants to low relative humidity. Am. J. Hort. Sci. 106: 515-518.

Chalupa, V. 1983. Micropropagation of conifer and broadleaved forest trees. Communicationes Instituti Forestalis Čechosloveniae13: 7-39. Forestry and Game Management Research Institute, Jílovi tě-Strnady ČSSR.

Conner, A.J. and M.B. Thomas. 1981. Re-establishing plantlets from tissue culture: a review. Comb. Proc. Int. Plant Prop. Soc. 31:342-357.

Davis, J.M. and D.E. Keathley. 1987. Differential responses to *in vitro* bud culture in mature *Robinia pseudoacacia* L. (black locust). Plant Cell Rep. 6:431-434.

Davis, T.D., B.E. Haissig and N. Sankhla. (eds.). 1988. Adventitious root formation in cuttings. Dioscorides Press, Portland, Oregon. 315 p.

Druart, P., C. Kevers, P. Boxus and T. Gaspar. 1982. *In vitro* promotion of root formation by apple shoots through darkness effect on endogenous phenols and peroxidases. Z. pflanzenphysiol. Bd. 108: 429-436.

Dunstan, D.I. and K. E. Turner. 1984. The acclimatization of micropropagated plants. In: Cell culture and somatic cell genetics of plants, Vasil, I.K. (ed.). Vol. 1, Laboratory procedures and their applications. Academic Press, New York. pp. 123-129.

Eliasson, L. 1978. Effects of nutrients and light on growth and root formation in *Pisum sativum* cuttings. Physiol. Plant. 43:13-18.

Gaspar, T. and M. Coumans. 1987. Root formation. In: Cell and tissue culture in forestry, Bonga, J.M. and D.J. Durzan (eds.). V.2. Martinus Nijhoff Publishers, Dordrecht. pp. 202-217.

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

Haissig, B. 1972. Meristematic activity during adventitious root primordium development. Plant Physiol. 49:886-892.

Haissig, B. 1974a. Influences of auxins and auxin synergists on adventitious root primordium initiation and development. N.Z.J. For. Sci. 4(2): 311-323.

Haissig, B. 1974b. Metabolism during adventitious root primordium initiation and development. N.Z.J. For. Sci. 4(2):324-37.

Hammerschlag, F. 1982. Factors influencing *in vitro* multiplication and rooting of the plum rootstock myrobalan (*Prunus cerasifera* Ehrh.). J. Amer. Soc. Hort. Sci. 107 (1):44-47.

Han, K. 1991. *Agrobacterium rhizogenes*- mediated transformation and plant regeneration from normal transgenic tissues of *Robinia pseudoacacia* L. Ph.D. Thesis, Michigan State University, East Lansing, Michigan. 96 p.

Hitchcock, A.E. and P.W. Zimmerman. 1939. Comparative activity of root-inducing substances and methods for treating cuttings. Contr. Boyce Thompson Inst. 10:461-480.

Hitchcock, A.E. and P.W. Zimmerman. 1940. Effects obtained with mixtures of root-inducing and other substances. Boyce Thompson Institute for Plant Research, Inc. Vol. II. pp. 143-160.

Hutchinson, J.F. and R.H. Zimmerman. 1987. Tissue culture of temperate fruit and nut trees. Hort. Rev. 9:273-349.

Hyndman, S., P.M. Hasegawa and R.A. Bressan. 1982. Stimulation of root initiation from cultured rose shoots through the use of reduced concentrations of mineral salts. HortScience 17(1):82-83.

James, D. 1983. Adventitious root formation *in vitro* in apple rootstocks (*Malus pumila*) I. Factors affecting the length of the auxin-sensitive phase in M.9. Physiol. Plant. 57:149-153.

Kato, M., H. Shibaoka and M. Shimokoriyama. 1978. The nature of the dual effect of auxin on root formation in *Azukian* cuttings. Plant and Cell Physiol. 19(8): 1535-1542.

Kawase, M. 1965. Etiolation and rooting in cuttings. Physiol. Plant. 18:1066-1076.

Khosh-Khui, M. and K.C. Sink. 1982. Rooting-enhancement of *Rosa hybrida* for tissue culture propagation. Scientia Horticulturae 17: 371-376.

Mullins, M. 1985. Regulation of adventitious root formation in microcuttings. Acta Horticulturae 166:53-61.

Nanda, K., P. Kumar and V. Kochhar. 1973. Role of auxins, antiauxins and phenol in the production and differentiation of callus on stem cuttings of *Populus robusta*. N.Z. J. For. Sci. 4(2):338-346.

Nemeth, G. 1986. Induction of rooting. In: Biotechnology in agriculture and forestry, Bajay, P.S. (ed.). Vol. 1: Trees I. Springer-Verlag. Berlin. pp. 49-64.

Sanchez, M.C. and A.M. Vieitez. 1991. *In vitro* morphogenetic competence of basal sprouts and crown branches of mature chestnuts. Tree Physiology 8: 59-70.

Schwarz, O.J. 1987. Plant growth regulator effects in the *in vitro* propagation of three hardwood tree genera: *Castanea, Juglans,* and *Quercus*. In: Hormonal control of tree growth, Kossuth, S.V. and S.D. Ross (eds.). Martinus Nijhoff Publishers. Dordrecht. pp. 113-135.

Scott, T.K. 1972. Auxins and roots. Annu. Rev. Plant Physiology 23:235-258.

Skoog, F. and W. Miller. 1957. Chemical regulation and organ formation in plant tissues cultured *in vitro*. Symp. Soc. Exp. Biol. 11:118-131.

Smith, D. and T. Thorpe. 1975. Root initiation in cuttings of *Pinus radiata* seedlings. I. Developmental sequence. J. Expt. Bot. 26(91):184-192.

Thompson, M.R. and T.A. Thorpe. 1987. Metabolic and non-metabolic roles of carbohydrates. In: Cell and tissue culture in forestry, Bonga, J.M. and D.J. Durzan (eds.). V.1. Martinus Nijhoff Publishers Group. Dordrecht. pp. 89-112.

Torrey, J.G. 1965. Physiological bases of organization and development in the root. In: Encyclopedia of plant physiology, Ruhland, W. (ed.). Vol. 15/1. Berlin, pp.1296-1327.

Vieitez, A.M. and M.L. Vieitez. 1982. *Castanea sativa* plantlets proliferated from axillary buds cultivated *in vitro*. Scientia Horticulturae 18:343-351.

Wetherell, D. 1982. Introduction to *in vitro* propagation. Avery Publishing Group Inc. New Jersey. pp. 69-73.

CHAPTER 3

Transformation of *Gleditsia triacanthos* L. (honeylocust)

ABSTRACT

The susceptibility of honeylocust to infection by *A. tumefaciens* and *A. rhizogenes* was demonstrated. Cotyledon and hypocotyl segments that were inoculated with bacterial suspensions developed tumors or hairy roots, depending on the type of inoculant. Internodal segments of root suckers and one month-old seedlings inoculated with *A. tumefaciens* suspension developed tumorous growth which enlarged as galls. The tumors from the cotyledon and hypocotyl segments and internodal segments of root suckers proliferated on MS medium without the addition of exogenous phytohormones.

Octopine was detected in the callus/tumor obtained from infected tissues but not in normal tissues. The autonomous callus/tumor growth and the presence of octopine in these tissues suggest amenability of honeylocust to transformation by *A. tumefaciens*. The results of the Southern blot analysis did not show physical evidence of the integration of the T-DNA into the honeylocust genomic DNA. It was recommended that in addition to the two restriction enzymes used in this study, more enzymes should be used to help find conclusive evidence of T-DNA integration into the honeylocust genome.

INTRODUCTION

The development of tumorous or rooty overgrowths on dicotyledonous plants is caused by the pathogenic soil bacteria *Agrobacterium tumefaciens* or *A. rhizogenes* (Tempe *et al.*, 1984; Zambryski, 1988; Zambryski *et al.*, 1989; Nester *et al.*, 1984). Formation of these growths involves gene transfer from the bacterial virulence plasmids to the plant (Bevan and Chilton, 1982), or the response of plant cells to the synthesis of plant hormones (Walden, 1989). The molecular basis for the pathogenicity is the transfer, integration and expression of a segment of the large Ti (tumor-inducing) or Ri (root-inducing) plasmid DNA into the nuclear genome of the host cell and its subsequent replication along with the host cell chromosomal DNA (Thomashow *et al.*, 1980a; Sciaky *et al.*, 1978; Nester *et al.*, 1981; Watson *et al.*, 1975; Yadav *et al.*, 1980). The specific part of the Ti plasmid known as T-DNA is transferred to the plant cell. This transfer is mediated by the virulence (vir) region of the Ti plasmid (Zambryski, 1992).

Some studies have focused on the structure of the T-DNA on the Ti plasmid and in the plant cell, the nature of the genes that it contains, the transcripts that it produces in the plant cell and its stability in the plant genome during whole plant regeneration and gametogenesis (Chilton *et al.*, 1978; Thomashow *et al.*, 1980). It has been shown that crown gall tumor tissues grow in axenic culture on a phytohormone-free medium (White and Braun, 1942). Expression of specific genes encoded by the T-DNA causes an alteration in the normal metabolism of auxins and cytokinins (Thomashow *et al.*, 1984) which are two classes of phytohormones generally required by plant tissues grown *in vitro* (Garfinkel *et al.*, 1981; Ooms *et al.*, 1981). Another important

phenotype of the plant tumor is the production of unusual compounds called opines (octopine, nopaline or agropine), which is specified by the genome of the bacterial strain that incited the tumor. In the natural environment opines provided by transformed plant cells supply the bacteria with utilizable carbon and nitrogen sources and exert a strong selective force favoring their development (Tempe *et al.*, 1984).

The natural ability of *Agrobacterium* to transfer T-DNA into plant cells, with its stable integration into the plant genome, is the basis for the use of the plasmid (Ti) as a vector in the transfer of foreign DNA into plant cells. By far it is the most successful transformation vector (Fincham and Ravetz, 1991). The Ti plasmids used as vectors are either disarmed i.e. the phytohormone biosynthetic genes are deleted from the T-DNA because they interfere with the protocols for the regeneration of plant tissues or the T-DNA may be completely recovered from the resident Ti plasmid (Gasser and Fraley, 1989). Gene transformation involves isolating a gene of interest, inserting it into the *Agrobacterium* T-DNA, genetically transforming the plant with DNA integrated into the nuclear genome, expression of the foreign gene in the recipient plant, regeneration of whole plants from the transformed cells and the possibility of the new trait being passed on to the progeny (Grierson and Covey, 1988; Parsons *et al.*, 1986). Another application of gene transfer is in fundamental research on the control of plant gene expression (Kuhlemeyer *et al.*, 1987; Schell, 1987).

The mechanism utilized by this pathogen to transform plant cells has been used most extensively to transfer a wide variety of foreign DNA's into the plant genome (Walden, 1989) and the first transgenic plants expressing engineered foreign genes were tobacco plants produced by the use of an *A. tumefaciens* vector (Horsch *et al.*,

1985). Most of the herbaceous dicots, all of the few woody dicots and a gymnosperm from which integration of foreign genes as well as species from which production of transgenic plants have been reported had utilized *A. tumefaciens and A. rhizogenes* (Ahuja, 1987; Parsons *et al.*, 1986; Guri and Sink, 1988; Han, 1991; Huang *et al.*, 1991; Fillatti *et al.*, 1987; McGranahan *et al.*, 1988; Davis and Keathley, 1989). No work on transformation in honeylocust has been reported.

Genetic engineering methods complement plant breeding efforts by increasing the diversity of genes and germplasm available for incorporation into crops and by shortening the time required for the production of new varieties and hybrids (Gasser and Fraley, 1989). The integration of biotechnology and breeding becomes more important and compelling for some woody species because of the long time between sexual generations which makes them well-suited for biotechnology, which in turn may shorten the genetic improvement process (Riemenschneider *et al.*, 1988).

Honeylocust is a moderately fast-growing tree and, due to its hardiness and tolerance to drought and salinity, it is widely planted for windbreak and soil erosion control (Blair, 1990). It is also planted as an ornamental tree for roadside and landscapes because of its beautiful foliage. It has been conceived as a desirable tree for agroforestry: pods provide fodder protein and carbohydrates, feathery foliage allows enough light penetration to support good pasture growth and wide adaptability for arable cropping systems (Smith, 1950; Bagley, 1976; Williams, 1982). An in-depth study by Gold (1984) was aimed at discerning the extent of genetic variation in honeylocust and demonstrating its potential for use as an agroforestry species. This study showed that honeylocust possesses a combination of desirable traits including

high wood specific gravity, abundant coppicing, a tap-rooted/profusely branched root system, drought tolerance, high carbohydrate pods and high protein seeds and leaves. Gold (1984) saw the need to overcome insect and disease problems in honeylocust. This species is a host to a number of leaf feeders: mites, moths, bugs, leaf hoppers, bark borers, scale insects, twig girdler, weevils and other damaging agents and among these, the most severe and widely distributed defoliator is the mimosa webworm (Blair, 1990). The development of gene transfer by *Agrobacterium* vectors offers interesting possibilities for producing honeylocust trees that are resistant to the insects.

The objective of this study was to demonstrate transformation of the honeylocust genome in both *in vitro* and *in vivo* cultures by showing infection response, expression of the transferred genes by formation of tumor and hairy roots and production of opines, specifically octopines, and the integration of the genes in the plant genome.

MATERIALS AND METHODS

Bacterial Cultures

Two strains each of *Agrobacterium tumefaciens* (A348 and A208) and *Agrobacterium rhizogenes* (R1601 and R1000) maintained as glycerol stocks at Michigan State University and previously used by Dr. John Davis (University of Florida) and some cultures (A348) by Dr. Kyung Huan Han (Oregon State University) were used. The bacteria were plated in Luria-Bertani (LB) medium solidified with 1.5% agar overnight at 30°C and single colonies were isolated and cultured overnight in 10 ml of LB in test tubes (20 x 150 mm) at 30° C and agitated at 70 rpm. These four strains were used in 1992 experiments whereas only A348 and R1601 were used in 1993, as these two strains were found to be the more virulent in the first experiment.

Preparation of Plant Materials for Inoculation

1. Cotyledons and hypocotyls. Seeds of *Gleditsia triacanthos* were obtained from the MSU Tree Research Center (TRC) and from a tree growing on the MSU campus beside the Biochemistry building in the early winter of 1992. Seed samples of two accessions (Calhoun, and Accession # 0477) were part of the seed collections of Gold (1984). The seeds were scarified and sterilized by soaking in 1.0% sodium hypochlorite solution containing five drops of Tween 20 surfactant for 15 minutes. The seeds were rinsed 4-5 times with sterilized double-distilled water and allowed to stand in the rinse for 30 minutes. They were then rinsed twice and transferred to germination medium. Two seeds were germinated per tube (20 x 150 mm) each

containing 5 ml of media with 5 g/l sucrose and 8 g/l agar and were incubated in a growth chamber at 26-27°C with a light intensity of 30-50 μMol/s/m^2 PAR. After 5-6 days, two cotyledonary disks measuring 6 mm were obtained using a a sterile hole punch and two hypocotyl segments measuring 12 mm in length were excised from each seedling.

2. Root sucker stem segments. Internodal segments (3-4 cm in length) were excised from root suckers from a honeylocust tree growing near the MSU library and were sterilized with 2.5% sodium hypochlorite solution for 20 minutes and washed 4x with sterilized double distilled water.

3. One month-old seedlings. Calhoun seeds were scarified as in (1) and washed under running water for 10 min. From 2-4 seeds were sown in seedboxes (10 cm x 10 cm) containing a peat/perlite/vermiculite (1:1:1) mixture. Nine boxes were contained in a plastic crate. They were maintained in the growth stand with the light intensity of 70-100 μMol/s/m^{2}PAR. Watering was done 4x per week.

Co-cultivation

1992 Experiment: Seeds of Calhoun and # 0477 were tested for infection. The cotyledonary disks and hypocotyl segments were dipped for ca. 3-5 minutes in sterile Petri dishes (100 x 15 mm) each containing 20 ml of bacterial suspension (4 strains), blotted in sterilized brown paper towels and cultured in Petri dishes each containing 20 ml of MS basal medium without phytohormones (MSO) solidified with 0.8% agar. Two cotyledonary disks and 2 hypocotyl segments from each seedling were tested for infection by each bacterial strain. Forty seedlings were tested from each of the two

accessions. All four bacterial strains were used. After 48 hours of incubation in the dark at 26 °C, the explants were transferred to fresh MSO medium containing 300 mg/l each of carbenicillin and cefotaxime antibiotics. Thereafter, the explants were sub-cultured every three weeks in MSO medium with the antibiotics. Tumor formation was scored on two month-old cultures. Presence/absence of tumors in cotyledons and hypocotyls was scored. From these data, the number of tumors formed were counted with the naked eye and the frequency of tumor formation was determined for each accession and each bacterial strain (expressed as number of seeds that formed tumors in the cotyledon or hypocotyl explants).

1993 Experiments: (1). Seeds of Calhoun and an MSU ornamental tree were tested. Two linear wounds across the diameter of the cotyledon disks (Figure 1A) or the length of the hypocotyl segments were made before dipping them for ca. 3-5 min. into the sterile Petri dish containing 20 ml of the bacterial culture. Forty seedlings were tested for infection by each strain. Only A348 and R1601 were used in this experiment, as these two strains were found to be the more virulent among the four strains initially used in 1992. (2). Internodal segments 3.5 - 4.0 cm in length were used as explants. Two linear wounds were made with a sterile scalpel on each explant placed on top of sterile paper towels before dipping in the bacterial suspension. Forty segments were tested, 4 per Petri dish. Strain A348 was used for inoculation. The inoculated explants were cultured in petri dishes containing the media as were used in 1992. Media for subcultures contained antibiotics as in 1992. Frequency of callus/tumor fomation was scored as in 1992.

For all cultures, subcultures were made every three weeks in a fresh medium containing antibiotics. During subculture, tumors attaining 15 mm or more in diameter were divided and necrotic tissues of the underlying part of the initial explants were removed with a sterile scalpel.

Infection of seedlings

1992 Experiments: One month-old Calhoun seedlings growing in waxed cardboard seedboxes (2 seedlings per box) containing peat:perlite:vermiculite mixture(1:1:1) were inoculated with the bacterial suspension by means of a 1-cc syringe. Four sites in each of three internodes above the cotyledonary node were wounded by pricking with a syringe while inoculating each site with ca. 0.1 cc of the bacterial suspension. Seedlings were inoculated with A348, A208, R1601 and R1500. Fifteen seedlings were tested for each strain. Tumor or hairy root formation was scored after two months.

1993 Experiments: One month-old Calhoun seedlings (4 seedlings sown/seedbox) were inoculated (Figure 1H). Only strain A348 was tested. Three types of wounding were tried: (1) wounding by pricking as in 1992, (2) wounding by fine incision, where in each of the three lower internodes above the cotyledonary node, two linear fine wounds were made with the syringe needle while inoculating with ca. 0.1 cc of the bacterial suspension, and (3) decapitation of the top of the seedling until ca. 2-3 nodes below the apex and inoculation of the cut end with ca. 0.2 cc of the bacterial suspension. Eighty seedlings were tested for each of the three methods.

Callus/tumor detection and scoring

Initial production of callus/tumors was detected with the aid of a hand lens. Eventually the galls and hairy roots grew and enlarged, therefore, the putatively transformed tissues could be seen with the naked eye. The cotyledon, hypocotyl and internodal segments were scored for the presence/absence of callus/tumors. From these data, the number of seedlings that formed at least one tumor on at least one cotyledon or hypocotyl was scored.

Opine Assay

Opines were assayed only from tissues presumed to be transformed by *A. tumefaciens* because although hairy roots were formed they failed to proliferate from explants inoculated with *A. rhizogenes*. Samples were not sufficient for opine analysis and DNA isolation.

Octopine analysis by paper electrophoresis was performed according to Otten and Schilperoort (1978) with some modifications. Approximately 0.5 g each of tumor samples initiated by *in vitro* and *in vivo* infection and normal calli were freeze-dried with liquid nitrogen, ground manually in a mortar and pestle and placed in microtubes with 1 volume of 95% ethanol and centrifuged at 12,000 x g for 5 minutes at 26 °C (Davis and Keathley, 1989). Five μl of the supernatant was spotted at the anodal region of a 3 MM Whatman Chromatography paper. Methylene green was used as a visual marker and 5 μl of authentic octopine and arginine were run simultaneously as controls.

Total DNA Isolation, Purification and Precipitation

The method to isolate total DNA of the tumor and calli was modified from and Chu (1992). Three grams each of fresh putatively transformed tumors, calli and normal calli were ground manually in liquid nitrogen in a pre-chilled mortar and pestle. The fine powder was suspended in 5 volumes of extraction buffer consisting of 50 mM Tris (pH 8.0), 10 mM EDTA, 1 M NaCl, 3 mM cysteine, 1 mM ascorbic acid, 50 μg/ml Proteinase K and of 5% N-lauroylsarcosine. The suspension was incubated for 3 hours at 55 °C and filtered through a nylon mesh. The suspension was centrifuged for 10 minutes at 5,500 rpm at 4 °C in a Sorvall HB-4 rotor. Then, 0.6 volume of isopropanol was added to the supernatant, the solution gently mixed and placed at 0 °C for 15 minutes. The mixture was centrifuged for 15 minutes in a Sorvall HB-4 rotor at 7,000 rpm at 4 °C. The supernatant was discarded and the pellets that remained as residue at the bottom of the plastic tubes were air-dried by inverting the tubes over filter paper. The pellets were dissolved in 2 ml of TE buffer, 6 ml of saturated CsCl and 50 μl of bisbenzimide and agitated in a shaker at 125 speed for one hour. The refractive index was checked at 1.396 - 1.397 range before subjecting to equilibrium density centrifugation for 14 hours using Sorvall TV-865 rotor at 42,000 x g at 19 °C.

DNA purification and precipitation after ultracentrifugation were modified from Stine (1988). The DNA bands were detected with UV light (366 nm), and DNA was collected with a glass pipette to which was added isopropanol saturated with NaCl solution and centrifuged in an Eppendorf centrifuge at 13,000 x g at 26 °C for 5 minutes. The topmost fluorescent region was removed as this contained isopropanol

and bisbenzimide. This step was repeated depending upon fluorescence that could still be detected after adding isopropanol.

For every volume of the CsCl-DNA, two volumes of double distilled water and three volumes of isopropanol were added and the DNA was precipitated at -70 °C for 20 minutes. The DNA mixture was later centrifuged in a microcentrifuge at 26 °C for 5 minutes and the supernatant was aspirated and discarded. Following this, 95% ethanol (1000 μl) was added to wash the DNA, which was then centrifuged in a microcentrifuge for three minutes and the supernatant again discarded. Washing of the DNA pellet was repeated using 70% alcohol and after centrifugation in a microcentrifuge alcohol was aspirated. The DNA pellets in the microtubes were air-dried by inverting the tubes over filter papers. The total DNA collected from the 3-gram weight of each kind of tissue (*in vitro* tumors, *in vivo* tumors and normal calli) was dissolved in 100 μl of TE buffer. The DNA was stored at 8 °C until further used for digestion and electrophoresis.

Digestion with Restriction Enzymes and Electrophoresis

DNA digestion was performed according to the specifications of the manufacturer of each enzyme (Eco RI and Bam HI) from Boehringer Mannheim Corporation (BMB). Lambda DNA used as a size marker was digested by Hind III (from BMB). DNA fragments were electrophoresed on 0.7% agarose gel (Sambrook *et al.*, 1989) and were run with the size marker and the probe DNA prepared as described below. After 15 hours of electrophoresis at 30 V, the DNA bands were visualized under the UV transilluminator and the gel was photographed using Polaroid film No. 55 or No. 57.

Southern Transfer

Following separation by gel electrophoresis, the digested DNA was transferred to Zeta-probe blotting membrane by DNA Capillary Transfer (BIO-RAD Chemical Division).

Probe Preparation

Two probes were used: Eco RI fragment 7 (6.9 kb cloned in PBR 325) and Bam HI fragment 8 (7.8 kb cloned in PBR 322), both from glycerol stocks of Dr. John Davis. The bacteria were plated in LB medium containing ampicillin at 100 mg/l overnight at 30 ^{0}C. Purified plasmid DNA from *E. coli* was obtained following the methods of Birnboim and Doly (1979) with some modifications. The specific restriction fragments used as probes were isolated from the vector DNA with the corresponding restriction endonuclease. These fragments were recovered after

electrophoretic separation by using S&S NA 45 DEAE membrane (Schleicher & Schleicher). The probes were ^{32}P-labeled by random prime labeling using Boerhringer Mannheim labeling kit.

Prehybridization, Hybridization and Autoradiography

Methods of Thomashow *et al.* (1980) and BIO-RAD were followed for prehybridization, hybridization and post-hybridization washing. The blot was placed in a polyethylene bag containing prehybridization solution consisting of 0.25 M NaH_2PO_4, ultra-pure 7% Sodium Doedecyl Sulfate and 1 mM EDTA and incubated at 65 °C overnight. The prehybridization solution was replaced with the same buffer and the denatured probe was added. Hybridization was set for overnight duration. After washing, the membranes were air-dried and wrapped between Saran Wrap and finally autoradiographed with X-ray film at -70 °C with two intensifying screens.

RESULTS and DISCUSSION

Ten days to two weeks after inoculation, small swellings developed on the smooth surface of the expanded cotyledons and at the cut margins. With the aid of a stereoscope at 10x magnification, calli were observed on cotyledons and hypocotyl explants inoculated with *A. tumefaciens*. Within 1-2 months calli and the small, smooth rounded tumors proliferated (Figure 1B). Regular three-week interval transfers to MSO medium with antibiotics maintained the growth and increased the size of the tumors. The bacteria persisted in a few explants until the third subculture. Tumors developing on the smooth surface of the cotyledons generally showed good growth and larger size than those found on the cut margins. Single gall/tumors later became aggregates of tumors of various sizes. Some infection responses which started as galls or tumors became open or loose and a mass of friable calli developed (Figure 1C). *Agrobacterium* strain A348 appeared more virulent (31% tumor formation) to both seed sources than A208 (5% tumor formation) which showed infection only in Calhoun (Table 1). This may be related to certain aspects of the plant receptor for attachment of *A. tumefaciens* which is a major factor in determining the host ranges of the bacterium (Matthysse, 1984). Some short-lived callus formation was observed in the wounded areas of the explants that were not inoculated. Hairy root formation was not induced by either strain of *A. rhizogenes* in the 1992 experiment. Two months from the start of the experiment ten explants were observed to have greenish spots within the tumors which appeared to be regenerating shoots. These areas were excised and transferred to MS medium containing 0.5 μM BAP and another with 0.2 μM zeatin with the same concentrations of the antibiotics used at the early part of the

early part of the experiment. Growth was slow - reaching ca. 10-15 mm in three months but eventually deteriorated (Figures 1D & E). There was a higher frequency of callus/tumor formation on cotyledons induced by A348 than hairy root formation by R1601 (Table 2). Hairy roots developing on cotyledon surfaces infected with R1601 in 1993 appeared dense and brown (Figure 1F). This may be related to the observation on roots hairs of honeylocust that become thick-walled and brown within a few days after they are produced in all sorts of habitats (McDougall, 1921). Unlike tumors induced by *A. tumefaciens*, the hairy roots did not survive further subcultures. Some nutrient components were probably required for proliferation and further growth.

Table 1. Response of cotyledon and hypocotyl explants after infection by co-cultivation with A348 and A208 (1992 experiment), n=40.

	Callus/tumor formation[1)]				
	Calhoun		Access. # 0477		
	Cotyl	Hypo	Cotyl	Hypo	Averages
Agro. strain					
A208	3/40(7.50)	5/40(12.5)	0/40(0)	0/40(0)	5%
A348	17/40(42.5)	10/40(25)	13/40(32.5)	9/40(22.5)	31%
Averages	Calhoun=22% Cotyl. =21%		Access. # 0477=14% Hypo.= 15%		

[1)] values of the numerator indicate no. of seeds forming tumors in the cotyledons or hypocotyl, denominator indicates initial no. of seeds infected. Values in parentheses are percentages.

Table 2. Response of cotyledon and hypocotyl explants after infection by co-cultivation with A348 and R1601 (1993 experiment), n=40.

	Callus/tumor formation or hairy root formation[1)]				
	Calhoun		MSU tree		
	Cotyl	Hypo	Cotyl	Hypo	Averages
Agro. strain					
A348	29/40(73)	14/40(35)	25/40(63)	8/40(20)	48%
R1601	10/40(25)	5/40(13)	8/40(20)	10/40(25)	21%
Averages	Calhoun 37% Cotyledon 45%		MSU tree 32% Hypocotyl 23%		

[1)] values of the numerator indicates no. of seeds forming tumors or hairy roots in the cotyledons or hypocotyl, denominator indicates initial no. of seeds used for infection. Values in parenthesis are percentages.

Root suckers inoculated with A348 showed roundish masses of calli. Green and white calli were observed (Figure 1G). With n=40, 35% formed tumors, 42.5% were apparently not contaminated by other microorganisms but had no tumor formation, and 22.5% were lost to contamination and browning of explants.

A similar response pattern was observed in one month-old seedlings (Figure 1H) inoculated *in vivo* by pricking while injecting the bacterial suspension with a syringe into the internodes. Tumors were visible after four weeks as small, roundish protuberances which enlarged as galls (Figure 1I) at the sites of infection. Ten and two seedlings from two different samples of size 15 of Calhoun developed tumors following inoculation with strain A348 and A208 respectively (Table 3). As with *in vitro* cultures, the honeylocust seedlings tested were not susceptible to the *A. rhizogenes* strains. Seedlings inoculated with A348 by either pricking or wounding by

fine incision showed that more than 50% developed tumors (Table 4). In these seedlings, part of stems that were located above the highest-formed tumors were usually thinner and of smaller diameters compared with the stems located in the lower part where the tumors were found. Stem areas which were near the sites of infection and which were not necessarily where the tumors were developing were swollen. Decapitated seedlings where tops were inoculated failed to form tumors.

Crown gall tumor resulting from the expression of genes in the *Agrobacterium* was shown in these studies. The oncogenic *Agrobacterium* transformation system used in this study was an appropriate and reliable method to detect evidence of the

Table 3. Response of honeylocust seedlings to inoculation with *A. tumefaciens* and *A. rhizogenes* (1992 experiment).

Strain	No. of Inoculated Seedlings	No. of Seedlings that Formed Tumors or Hairy Roots
A348	15	10
A208	15	2
R1601	15	0
R1000	15	0

Table 4. Response of one month-old seedlings to wounding and inoculation with *A. tumefaciens* (1993 experiment).

Strain	Type of Wounding	No. of Inoculated Seedlings	No. of Seedlings that Developed Tumors
A348	Pricking	80	52
	Fine Incision	80	53
	Decapitation	80	0

susceptibility of honeylocust to infection by the *Agrobacterium*. The transformed cells were easily detected because the tumors and calli emerged as protuberances from the inoculated organs. It is, therefore, a useful system for a quick detection of putative transformation. The calli that developed from infected explants cultured *in vitro* showed autonomous phytohormone growth through several sub-cultures. The incisions made on the cotyledons and stems of root suckers during co-cultivation and in the stems of intact seedlings when they were inoculated with bacteria increased the amount of transformed cells. The cotyledons and root sucker stem segments were very responsive and competent for *Agrobacterium* transformation by forming callus and tumors after infection compared with the hypocotyls. This could reflect qualitative and quantitative differences in endogenous phytohormone production between cotyledons and hypocotyls (Schilperoort *et al.*, 1978). This could also explain the response of the root sucker stem segments. These explants were also responsive to shoot induction and elongation, as was found in the experiment given in

Chapter 1. In addition, incisions made along the length of the hypocotyls increased the amount of brownish exudates, probably phenolic compounds, which were found to be inhibitory to callus/tumor formation at the site of infection.

The transformed cells in the calli and tumors that proliferated on MSO without the addition of exogenous phytohormone showed potential for shoot regeneration. A few regenerating shoots reached 1.0 -1.5 cm in height after three months in culture but were not morphologically normal and failed to elongate further. This could be due to improper balance of auxins and cytokinins whose production is encoded by the oncogenic genes in the inserted T-DNA (Thomashow *et al.*, 1984). A suitable auxin/cytokinin balance in the medium needs to be found for regeneration of transformed tissues.

Octopine detected in the callus/tumors from infected plants but not from normal callus gave further evidence for the expression of foreign genes. As seen in the plasmid map (Figure 2), the gene for opine production is located toward the right end of the T_L-DNA. Thus, the gene for opine production appeared to have been inserted also. Paper electropherogram showing the presence of opines in putatively transformed tissues are given in Figures 3 & 4. The Ti plasmid present in strain A348 is pTiA6 which encodes octopine production (Binns and Thomashow, 1988). Other Ti plasmids encode nopaline production in crown galls. Octopine and nopaline crown gall tumors contain a foreign DNA (T-DNA) which is homologous and colinear with a defined fragment of the corresponding Ti-plasmid present in the tumor - inducing bacteria (Chilton *et al.*, 1977). Most tumors have the ability to synthesize

octopine, an amino acid derived by a condensation of arginine and pyruvate that is not otherwise found in plant tissues (Lichtenstein and Draper, 1985). Crude ethanol extracts of tumor or calli show octopine in lanes 2 and 3 of both figures (Figures 3 & 4) corresponding to the octopine standards in lanes 1 and 6. Callus from uninoculated tissues in lanes 5 of both figures did not exhibit octopine. Also in Figures 3 & 4, transformed and nontransformed tissues show co-migration with the arginine standard spotted in lanes 1 and 4.

Only a particular segment of the Ti-plasmid DNA called T-DNA is stably integrated in the plant genome (Thomashow *et al.*, 1980). In octopine Ti plasmid like the pTiA6 present in A348, there are two regions called T_L (T-left) and T_R (T-right) which can be transferred and inserted independently into the plant genome (Thomashow *et al.*, 1980). The T_L DNA is responsible for the tumorous state of the plant cells as it encodes essential functions involved in the neoplastic transformation of plant cells (Garfinkel *et al.*, 1981; Willmitzer *et al.*; 1982). The genes code for enzymes for the production of auxins and cytokinins at the *tms* and *tmr* loci respectively (Gelvin *et al.*, 1981; Wilmitzer *et al.*, 1982). These loci are seen in the physical map of the T_L fragment of pTiA6 (Figure 2). Figure 5 shows the Southern blot of DNA isolated from the tumors incited by A348 and normal callus as well as from the plasmids used as probes. The probe Eco RI 7 from the pTiA6 plasmid was expected to hybridize with four fragments from the Bam HI digested T-DNA and to a single 6.9 kb fragment from Eco RI digested T-DNA. Lanes 1 and 4 from undigested tumor DNA showed faint hybridization. The uppermost dark bands ca. 7.9 kb and 6.9 kb in Lanes 2 and 5 respectively (digested by Bam HI)) indicate hybridization of

the probe with the fragments. These bands are the Bam 8 bands. The third lower dark bands (ca. 4.7 kb) in Lanes 2 and 5 could be fragment 19. A lighter band between fragments 8 and 19 and which is ca. 5.5 kb could be a partial digest of fragment 19. A faint band ca. 1.65 kb could be fragment 29.

Lanes 3 and 6 are extracts digested by Eco RI from *in vitro* tumors and intact seedlings respectively. The probe Eco RI fragment 7 hybridized to the expected single 6.9 kb Eco RI fragment 7. There appeared a faint band above this fragment which could be a partial digest. Lanes 8 and 9 which is DNA from normal callus digested by Bam 8 and Eco RI did not show hybridization with the probe.

Figure 6 is a Southern blot of the DNA from normal and tumor tissues probed by Bam 8. The expected hybridization is seen between the 7.8 kb Bam 8 probe and Bam H1 digested fragment 8 in lanes 2 and 5. Another expected hybridization is seen between the probe used and Eco RI fragment 7 in lanes 3 and 6. A faint band (ca. 8.5 kb) behind Bam 8 fragment in lane 5 is probably a partial digest of Bam 8 fragment.

The demonstration of phytohormone autonomous growth and octopine production suggests that honeylocust is amenable to *A. tumefaciens* - mediated transformation. The occurrence of a few regenerating shoots from the putatively transformed tumors from infected cotyledons suggests a strong possibility for the production of transgenic honeylocust via *A. tumefaciens* vector. These shoots grew slowly and deteriorated which may be due to imbalance of auxins and cytokinins whose production in tumorous tissues, as already mentioned earlier is known to be encoded by the *onc* genes in the transferred DNA (Thomashow *et al.*, 1984; Walden, 1989). Root sucker

segments were also responsive to the transformation method used in this study by showing profuse, vigorous calli. Plantlets were produced by using this type of explants in micropropagation experiments (Chapter 1). These responsive explants which came from adult trees, therefore, are expected to be important components of a strategy for future transformation in honeylocust and multiplication of trees with desirable traits.

There are two pieces of evidence that suggest the presence of T-DNA gene sequences in the honeylocust genome. The first is the continued growth and proliferation of the tumors on MSO (this would normally require exogenous auxins and cytokinins) after the bacteria were eliminated and second is the presence of octopine in the tumors and none in normal tissues. It has been generally considered that opine biosynthesis by plants is a crown gall (or hairy root) tumor-specific process and there are several reports that opines could not be detected in untransformed tissues (Lichtenstein and Draper, 1985; Holderbach and Beiderbeck, 1976), although Christou *et al.*, (1986) reported opine synthesis in callus and normal tissues of soybean and cotton. Based on the relative sizes of the restriction fragments of the tumor DNA that hybridized with the probes (Figures 5 & 6, lanes 2,3,5 & 6) the restriction enzymes appeared to cut within the T-DNA only and therefore did not show physical evidence of the T-DNA integrating into honeylocust genomic DNA. However, if the first honeylocust Eco R1 and Bam H1 sites are very close to the site of the T-DNA integration, the size of the restriction fragment cut by these enzymes may not be large enough to detect the integration event. Nevertheless, the overall results of the Southern blot analysis did not exclude the possibility that the T-DNA

was not integrated into the honeylocust genome. Therefore, although the two main physiological- biochemical evidences - phytohormone independence in tumor growth and presence of octopine were obtained, in addition to the fact that axenic tumors which were subdivided and subcultured repeatedly were obtained, and which continued to proliferate, Southern analysis could not conclusively confirm the integration of T-DNA into the honeylocust genome. Further research in this direction will require substantial amount of putatively transformed tumor samples and the use of several restriction enzymes whose restriction sites within the T-DNA are known. It is useful to include enzymes that cut twice within the plasmid, an enzyme that cuts once within the plasmid and an enzyme that does not cut within the plasmid to distinguish between the free plasmid and the integrated one (Jenes *et al.*, 1993).

In the future, transformation of honeylocust cells should be tried using non-oncogenic vectors carrying marker genes which are linked to the foreign gene that is to be introduced. This should be possible since the *onc* genes encoded by the Ti plasmid are neither required for the transfer of the T-DNA to the plant cell nor its integration into the nuclear DNA so they may be replaced by the foreign DNA to be inserted (Armitage *et al.*, 1988). Thus, with this gene transfer system, a gene of interest can be inserted into the modified T-DNA and transferred to honeylocust.

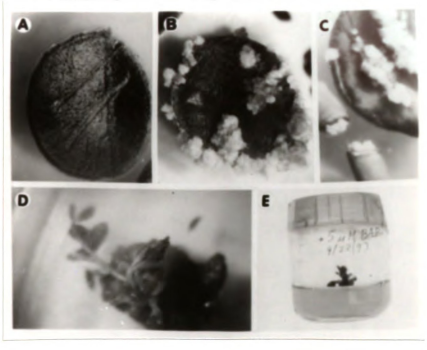

Figure 1. Infection of cotyledon disks, hypocotyl segments and root sucker stem segments with *Agrobacterium tumefaciens* and *A. rhizogenes*. **(A)** cotyledon disks with two linear wounds prepared for inoculation. **(B)** Tumor and callus proliferation on cotyledon disks inoculated with A348. **(C)** Calli becoming more friable in cotyledon disks and hypocotyl segments. **(D)** & **(E)** Regenerated shoot from A348-incited tumor.

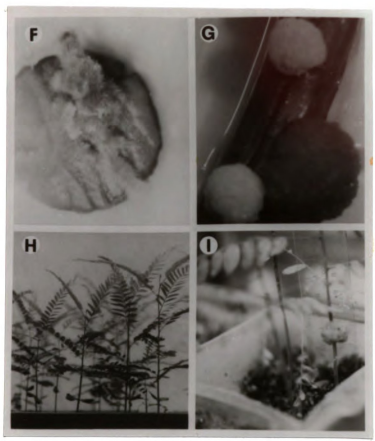

Figure 1 (Cont'd). **(F)** Hairy root incited by R1601. **(G)** White and green compact calli on nodal segments or root suckers inoculated with A348. **(H)** One month-old honeylocust seedlings inoculated with A348 by injection. **(I)** Five month-old gall in seedling inoculated with A348.

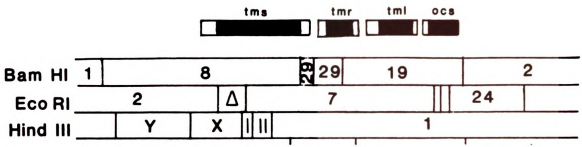

Figure 2. Partial restriction map of the T_L-fragment of pTiA6 (Nester *et al.*, 1984)

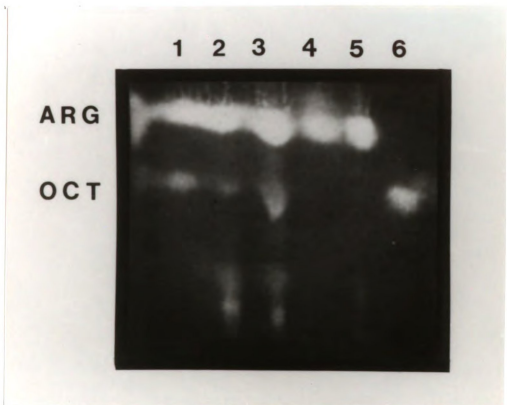

Figure 3. Paper electropherogram showing the presence of octopine in tumors incited by A348 in cotyledon and hypocotyl tissues. **Lane 1.** arginine and octopine standards. **Lane 2.** extracts from tumors of cotyledon. **Lane 3.** extracts from tumors of hypocotyl. **Lane 4.** authentic arginine. **Lane 5.** extracts from non-transformed callus. **Lane 6.** octopine standard.

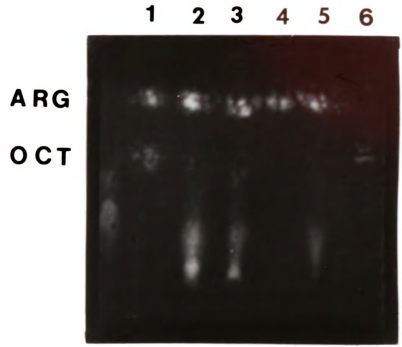

Figure 4. Paper electropherogram showing the presence of octopine in tumors incited by A348 in explant segments of root sucker and stems of intact seedling. **Lane 1.** arginine and octopine standards. **Lane 2.** extracts from tumors of root sucker. **Lane 3.** extracts from tumors of seedling. **Lane 4.** authentic arginine. **Lane 5.** extracts from non-transformed callus. **Lane 6.** authentic octopine.

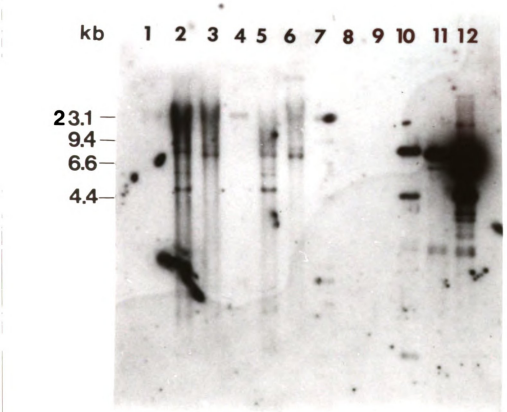

Figure 5. Southern blot analysis of transformed honeylocust tumors. DNA's were digested with Eco RI and Bam HI. The Southern blot was probed with ^{32}P-labeled Eco RI fragment 7. **Lane 1.** Undigested tumor DNA from cotyledon. **Lane 2.** Cotyledon tumor DNA digested by Bam HI. **Lane 3.** Cotyledon tumor DNA digested by Eco RI. **Lane 4.** Undigested tumor DNA from seedling. **Lane 5.** Seedling tumor DNA digested by Bam HI. **Lane 6.** Seedling tumor DNA digested by Eco RI. **Lane 7.** Lambda DNA. **Lane 8.** Callus DNA from non-transformed cotyledon. **Lane 9.** Callus DNA from non-transformed hypocotyl. **Lane 10.** Bam HI fragment 8 probe. **Lane 11.** Eco RI fragment 7. **Lane 12.** Eco RI plasmid. On the left are size markers based on DNA digestion by Hind III.

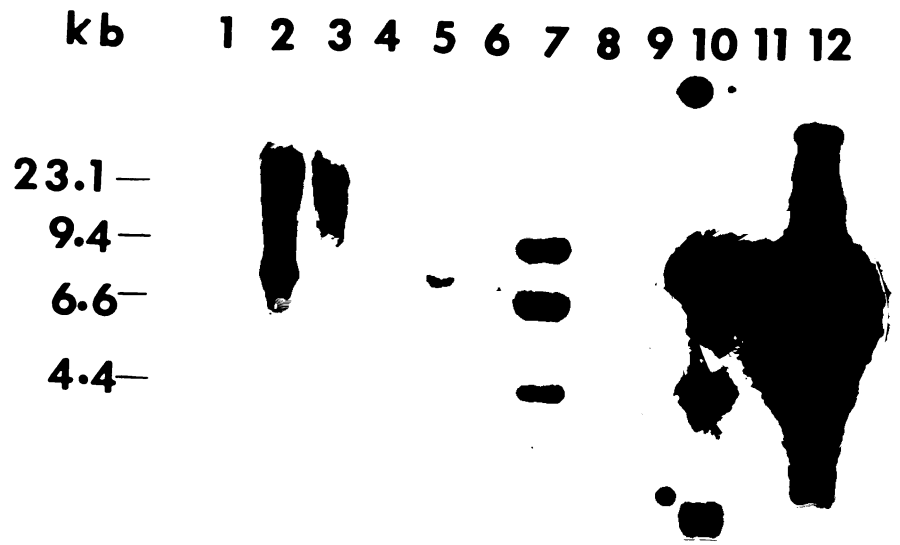

Figure 6. Southern blot analysis of transformed honeylocust tumors. DNA's were digested with Eco RI and Bam HI. The Southern blot was probed with ^{32}P-labeled Bam HI fragment 8 probe. **Lane 1.** Undigested tumor DNA from cotyledon. **Lane 2.** Cotyledon tumor DNA digested by Bam HI. **Lane 3.** Cotyledon tumor DNA digested by Eco RI. **Lane 4.** Undigested tumor DNA from seedling. **Lane 5.** Seedling tumor DNA digested by Bam HI. **Lane 6.** Seedling tumor DNA digested by Eco RI. **Lane 7.** Lambda DNA. **Lane 8.** Callus DNA from non-transformed cotyledon. **Lane 9.** Callus DNA from non-transformed hypocotyl. **Lane 10.** Bam HI fragment 8 probe. **Lane 11.** Eco RI fragment 7 probe. **Lane 12.** Eco RI plasmid. On the left are size markers based on DNA digestion by Hind III.

LITERATURE CITED

Ahuja, M. R. 1987. Gene transfer in forest trees. In: Genetic manipulation of woody plants, Hanover, J. and D. Keathley (eds.). Plenum Press, New York . pp. 25-41.

Armitage, P. R. Walden and J. Draper. 1988. Vectors for the transformation of plant cells using *Agrobacterium*. In: Plant genetic transformation and gene expression, Draper, J., R. Scott and P. Armitage, (eds.). A laboratory manual. Blackwell Scientific Publications. pp. 3-67.

Bagley, W. T. 1976. Multipurpose tree plantations. In: Shelterbelts on the Great Plains. Great Plains Ag. Council Publ. No. 78. Proc. of the Symp. Denver, April 20-22. pp. 125-128.

Blair, R. 1990. *Gleditisa triacanthos* L. Honeylocust. In: Silvics of North America. V. 2. Hardwoods. R. M. Burns and B. Honkala, (Tech. Coordinators). Forest Service, United States Department of Agriculture. pp. 358-364.

Bevan, M. W. and M. D. Chilton. 1982. T-DNA of the *Agrobacterium* Ti- and Ri- plasmids. Annu. Rev. Genet. 16:357-84.

Binns, A. N. and M. Thomashow. 1988. Cell biology of *Agrobacterium* infection and transformation of plants. Annu. Rev. Microbiol. 42:575- 606.

Birnboim, H. C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid 7:1513-1523.

Chilton, M. D., M. H. Drummond, D. M. H. Merlo, and D. Sciaky. 1978. Highly conserved DNA of Ti -plasmids overlaps T-DNA maintained in plant tumors. Nature 275:147-49.

Chilton, M. D., M. H. Drummond, M. H. Merlo, D. Sciaky, A. L. Montoya. 1977. Stable incorporation of plasmid DNA into higher plants: The molecular basis of crown gall tumorigenesis. Cell 11:263-71.

Chu, C. 1992. A system to study plastid development in spruce. Ph.D. Thesis. Michigan State University, USA. 79p.

Christou, P., S. Platt, and M. Ackerman. 1986. Opine synthesis in wild-type plant tissue. Plant Physiol. 82:218-221.

Davis, J. M. and D. E. Keathley. 1989. Detection and analysis of T-DNA in crown gall tumors and kanamycin-resistant callus of *Robinia pseudoacacia.* Can. J. For. Res. 19: 1118-1123.

Fillatti, J.J., J. Sellmer, B. McCown, B. Haissig, and L.. Comai. 1987. *Agrobacterium*-mediated transformation and regeneration of *Populus*. Mol. Gen. Genet. 206:192-199.

Fincham, J. R. S. and J. R. Ravetz. 1991. Genetically engineered organisms. University of Toronto Press, Toronto, Buffalo. pp. 48-68.

Garfinkel, D. J., R. B. Simpson, L. W. Ream, F. F. White, P.Gordon, and E. Nester. 1981. Genetic analysis of crown gall: Fine structure map of the T-DNA by site-directed mutagenesis. Cell 27:143-53.

Gasser, C. and R. Fraley. 1989. Genetically engineering plants for crop improvement. Science 244:1293-1299.

Gelvin, S. B., M. P. Gordon, E. W. Nester and A. A. Aronson. 1981. Transcription of the *Agrobacterium* Ti- plasmid in the bacterium and in crown gall tumors. Plasmid 6: 17-29.

Gold, M. A. 1984. Honeylocust (*Gleditsia triacanthos* L.): Genetic variation and potential use as an agroforestry species. Ph.D. Thesis, Michigan State University, East Lansing, Michigan. 163 p.

Grierson, D. and S. Covey. 1988. Plant molecular biology. Chapman and Hall New York. pp. 142-57.

Guri, A. and K. C. Sink. 1988. *Agrobacterium* transformation of eggplant. J. Plant Physiology 133:52-55.

Han, K. 1991. *Agrobacterium rhizogenes* - mediated transformation and plant regeneration from normal transgenic tissues of *Robinia pseudoacacia* L. Ph.D.Thesis, Michigan State University, East Lansing, Michigan. 96 p.

Holderbach, F. and R. Beiderbeck. 1976. Octopingehalt in normal-und Tumor-geweben eineger höherer Pflanzen. Phytochemistry 15:955-956.

Horsch, R. B., J. E. Fry, N. L. Hoffmann, D. Eichholtz, S. G. Rodgers and R. T. Fraley. 1985. A simple and general method for transferring genes into plants. Science 227:1229-31.

Huang, Y., A. Diner, and D. Karnosky. 1991. *Agrobacterium rhizogenes* - mediated genetic transformation and regeneration of a conifer: *Larix decidua*. *In Vitro* Cell Dev. Biol. 27:201-207.

Jenes, B., H. Moore, J. Cao, W. Zhang, and R. Wu. 1993. Techniques for gene transfer. In: Transgenic plants, Kung S. and R. Wu (eds.). Academic Press, Inc., San Diego, Ca. pp. 125-146.

Kuhlemeyer, C., P.J. Green and N.H. Chua. 1987. Regulation of gene expression in higher plants. Ann. Rev. Plant Physiol. 38:221-257.

Lichtenstein, C. and J. Draper. 1985. Genetic engineering of plants. In: DNA Cloning, Clover, D. M. (ed.). V.II. IRL Press, Oxford. Washington D. C. pp. 67-119.

Matthysse, A. G. 1984. Interaction of *Agrobacterium tumefaciens* with the plant cell surface. In: Genes involved in microbe-plant interaction, Verma, D. and Th. Hohn (eds.). Springer-Verlag Wien New York. pp. 33-54.

McDougall, W.B. 1921. Thick-walled root hairs of *Gleditsia* and related genera. Amer. J. Bot. 8:171-175.

McGranahan, G. H., C. A. Leslie, S. L. Uratsu, L. A. Martin and A. M. Dandekar. 1988. *Agrobacterium* -mediated transformation of walnut somatic embryos and regeneration of transgenic plants. Bio/technology 6:800-4.

Nester, E., M. Gordon and T. Kosuge. 1981. Plasmids specifying plant hyperplasias. Annu. Rev. Microbiol. 35:531-65.

Nester, E., M. Gordon, R. Amasino, and M. Yanofsky. 1984. Crown gall: A molecular and physiological analysis. Annu. Rev. Plant Physiol. 35: 387-413.

Ooms, G., P. J. Hooykaas, G. Moolenaar and R. A. Schilperoort. 1981. Crown gall plant tumors of abnormal morphology, induced by *Agrobacterium tumefaciens* carrying mutated octopine Ti plasmids: Analysis of T-DNA functions. Gene 14:33-50.

Otten, L. A. and R. A. Schilperoort. 1978. A rapid microscale method for the detection of lysopine and nopaline dehydrogenase activities. Biophys. Acta 527:497-500.

Parsons, T. J., V. Sinkar, R. Stettler, E. Nester and M. Gordon. 1986. Transformation of poplar by *Agrobacterium tumefaciens*. Biotechnology 4:533-535.

Riemenschneider, D. E., B. E. Haissig, and E. T. Bingham. 1988. Integrating biotechnology into woody plant breeding programs. In: Genetic manipulation of woody plants, Hanover, J. W. and D. E. Keathley, (eds.). Plenum Press, New York. pp. 433-449.

Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecular cloning. A laboratory manual. Second ed. Cold Spring Harbor Laboratory Press, USA. pp. 6.3-6.9.

Schell, J. 1987. Transgenic plants as tools to study the molecular organization of plant genes. Science 237: 1176-1183.

Schilperoort, R., A. P. M. Klapwijk, P. J. J. Hooykaas, B. P. Koekman, G. Ooms, L. Otten,. E. M. Wurzer-Figurelli, G. J. Wullems, A. Rorsch. 1978. *A. tumefaciens* plasmids as vectors for genetic transformation of plant cells. In: Frontiers of plant tissue culture. Thorpe, T. A. (ed.). International Association for Plant Tissue Culture, Calgary Univ. pp. 85-94.

Sciaky, D., A. L. Montoya, and M. D. Chilton. 1978. Fingerprints of *Agrobacteriumn* Ti plasmids. Plasmid 1:238-252.

Smith, J. R. 1950. Tree crops. A permanent agriculture. The Devin-Adair Company, New York. pp. 65-79.

Stine, M. 1988. Demonstration of paternal inheritance of plastids in *Picea* (Pinaceae). Ph.D. Thesis. Michigan State University, East Lansing, Michigan, 63 pp.

Tempe, J., A. Petit and S. Farrand. 1984. Induction of cell proliferation by *Agrobacterium tumefaciens and A. rhizogenes*: a parasite's point of view. In: Genes involved in microbe-plant interactions, Verma, D. P. S. and Th. Hohn, (eds.). Springer-Verlag Wien New York. pp. 272-286.

Thomashow, M., F., R. Nutter, A. L. Montoya, M. P. Gordon and E. W. Nester. 1980. Integration and organization of Ti plasmid sequences in crown gall tumors. Cell 19: 729-39.

Thomashow, L. S., A. Reeves and M. Thomashow. 1984. Crown gall oncogenesis: Evidence that a T-DNA gene from the *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyzes synthesis of indoleacetic acid. Proc. Natl. Acad. Sci. USA 81:5071-5075.

Walden, R. 1989. Genetic transformation in plants. Prentice Hall, New Jersey. 138 p.

Watson, B., T. C. Currier, M. P. Gordon, M. Chilton, and E. Nester. 1975. Plasmids required for virulence of *Agrobacterium tumefaciens*. J. Bacteriol. 123:255-64.

White F. and A. C. Braun. 1942. A cancerous neoplasm of plants. Autonomous bacteria-free crown gall tissue. Cancer Res. 2, 597-617.

Williams, G. 1982. Energy conserving perennial agriculture for marginal land in Southern Appalachia. Final Tech. Report to Dept. of Energy Appropriate Technology Small Grants Program. 37 p.

Willmitzer, L., G. Simons and J. Schell. 1982. The T_L-DNA in octopine crown gall tumors codes for seven well-defined polyadenylated transcripts. EMBO J. 1:139-46.

Yadav, N. S., K. Postle, R. K. Saiki, M. F. Thomashow and M.-D. Chilton. 1980. T-DNA of a crown-gall teratoma is covalently joined to plant DNA. Nature 287: 458-461.

Zambryski, P. 1988. Basic processes underlying *Agrobacterium* -mediated DNA transfer to plant cells. Annu. Rev. Genet. 22:1-30.

Zambryski, P., J. Tempe, and J. Schell. 1989. Transfer and function of T-DNA genes from *Agrobacterium* Ti and Ri plasmids in plants. Cell 56:193-201.

Zambryski, P. 1992. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43:465-90.

CONCLUSIONS AND RECOMMENDATIONS

Tissue culture techniques used in this research study produced plants from explants of germinating seedlings, 8 & 10 month-old seedlings, juvenile and mature honeylocust trees.

Among four BAP levels (μM) tested (5.0. 10, 15 and 30), 30 μM induced the highest percentage of shoot bud formation. Shoot buds elongated when subcultured in media containing 1 μM BAP.

There was a differential response to 1.0 μM BAP of buds at various positions in 10 month-old seedlings, with buds occupying the sixth position upward until the sixteenth node above the cotyledonary node giving significantly higher elongation percentage than nodes in the second or fourth positions nearer the root.

The study showed that micropropagation using root suckers and stump sprouts is more feasible than the use of dormant buds and nodal explants of summer elongating crown shoots. Application of cultural practices such as wounding that stimulate the development of root sprouts would make available numerous explants for further research. Hormonal treatments should be sought to induce multiple shoot formation in the regenerated shoots of root suckers and stump sprouts.

Shoots (2 cm or longer) that regenerated from cotyledon nodes and hypocotyl segments as well as shoots from root suckers and stump sprouts and 10 month-old seedlings rooted when cultured in the dark for 9 days in a modified MS medium containing one-half strength nitrates, 10 g/l sugar and supplemented with IBA (2 mg/l), or NAA (1 mg/l or 2 mg/l) or a mixture of 1 mg/l IBA and 1 mg/l NAA and subcultured in an auxin-free medium in an alternating 16 hr. light and 8 hr. dark

lighting regime after the dark pretreatment. As to which factor or factors were critical could be studied with more shoots available for experimentation. The length of the dark period for optimal rooting should also be ascertained for honeylocust.

The plantlets acclimatized in a peat:perlite:vermiculite (1:1:1) mixture in a moist environment. Nineteen plantlets from germinating tissues and sixteen plantlets from adult trees reached average height of 66 cm and 61 cm respectively in eight months.

There is no report so far found in the literature concerning genetic transformation of honeylocust. The results obtained in this study showed evidence for the feasibility of *Agrobacterium tumefaciens*-mediated transformation in this species. These evidences came from the resulting phytohormone independent growth of proliferating calli incited by the bacterium, and the presence of opines in these tissues which are among transformation markers. Southern analysis did not conclusively confirm the integration of T-DNA into the honeylocust genome. Thus, in addition to the two restriction enzymes used, it will be useful to include more enzymes to provide proof for integration of the foreign gene (Jenes *et al.*, 1993). A second step in future research would be the use of non-oncogenic vectors carrying marker genes which are linked to the foreign gene that is to be introduced. The use of root sucker internodal segments should be tried in future attempts for developing methods for the regeneration of whole plantlets from unorganized tissues.

Literature Cited

Jenes, B., H. Moore, J. Cao, W. Zhang, and R. Wu. 1993. Techniques for gene transfer. In: Transgenic plants, Kung S. and R. Wu (eds.). Academic Press, Inc., San Diego, Ca. pp. 25-146.

APPENDICES

Appendix 1. Friedman two-way analysis of variance of the response of honeylocust dormant buds to MS medium containing varying BAP levels and NAA in MS medium varying in carbon sources.

BAP levels (μM)	Rank sum
(1) 1.0	45.500
(2) 3.2	47.500
(3) 5.0	27.50

Friedman test statistic = 11.775*
k (no. of treatments) =3
n (no. of blocks) = 5
α = 0.05, P=0.003
Critical χ^2 value =5.99
Conclusions: * exceeds the critical value, null hypothesis that the effects of BAP levels on greenness frequency are all the same, is rejected.

Multiple comparisons of treatments (BAP levels) based on Friedman rank sums
Rank sum differences (T_p - T_q)

BAP levels	1	2	3
1		1.5	18.0*
2			19.5*
3			

k (no. of treatments) =3, n (no. of blocks) =5, α= .039 (critical value=8), α = .008 (critical value=9).
* higher than the critical values, effects of BAP levels 1 & 3, 2 & 3 are not significantly equal to zero.

Appendix 2. Friedman two-way analysis of variance of the response of honeylocust dormant buds to MS medium containing varying BAP and NAA levels, solidified by two concentrations of bacto-agar.

BAP levels (μM)	Rank Sum
1.0	49.50
3.2	44.00
5.0	26.50

Friedman test statistic= 14.425. The value of $\chi^2_{.05}$ for T(treatment) -1=2 and B (blocks) - 1 =2 is 5.99. Since the calculated value of Friedman test statistic is greater than the tabular value of $\chi^2_{.05}$= 5.99, the Null hypothesis of no difference in effects on greenness frequency among the BAP levels is rejected.

Multiple comparison of treatments based on Friedman rank sums
Friedman rank sum differences (T_p- T_q)

BAP levels	1	2	3
1		5.5	23*
2			17.5*
3			

k (treatment) = 3, blocks = 5(α = 0.039, critical value = 8), (α = .008, critical value=9).

* These values exceed the critical value; null hypothesis of equal effects of p and q treatments (BAP levels) on greenness frequency is rejected.

Appendix 3. Kruskal-Wallis one-way ANOVA for bud breaking in root suckers. Treatment (Tmt.)1= plus 0.1 μM GA, Tmt. 2=plus 0.3 μM GA, Tmt. 3=plus 1.5 μM GA.

Treatment	Budbreak percentage	Replication	Rank sum	Average rank
1	61	14	309.50	22
2	78	11	253.50	23
3	51	12	140.00	12

Kruskal-Wallis test statistic= 9.226
Probability is 0.01 assuming Chi-square distribution with 2 df

Pairwise comparison:

Tmt.	Difference	Statistic
(1&2)	0.93	10.42
(1&3)	10.44 *	10.17
(2&3)	11.37 *	10.79

*greater than computed statistic, therefore pairs of treatments are significantly different