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Agrobacterium Rhizogenes-Mediated Transformation and Plant Regeneration from Normal and Genetically Transformed Tissues of Robinia Pseudoacacia L.

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Kyung-Hwan Han

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Daniel E. Keathley
Major professor

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Agrobacterium rhizogenes-MEDIATED TRANSFORMATION AND PLANT REGENERATION FROM NORMAL AND TRANSGENIC TISSUES OF *Robinia pseudoacacia* L.

By

Kyung-Hwan Han

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ABSTRACT

Agrobacterium rhizogenes-MEDIATED TRANSFORMATION AND PLANT REGENERATION FROM NORMAL AND TRANSGENIC TISSUES OF *Robinia pseudoacacia* L.

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The objectives of this study was to develop the system for introduction of foreign genes into black locust genome. A prerequisite was the need for stable methods for regeneration of whole plants in order for genetic manipulation at the cellular and molecular levels to be utilized in a tree improvement program.

To devise the regeneration system for callus induction and subsequent shoot regeneration, various tissue sources such as hypocotyl, internodal segments of *in vitro* shoot cultures, and cambial tissues of mature black locust were used in the experiments described in the first and second chapters of this dissertation. Calli from both juvenile and mature sources were successfully regenerated into whole plants.

For the development of gene transfer system, hypocotyl segments were co-cultivated with *Agrobacterium rhizogenes* strains harboring Ri-plasmid in which kanamycin resistance gene has been inserted. Subsequent hairy root formation was observed on explants cultured on phytohormone-free medium. Spontaneous shoot regeneration occurred on less

than 5% of the hairy roots. Transformed shoots were maintained and rooted in the presence of 100 ug/ml kanamycin. All transformants showed hairy root phenotype characterized by wrinkled leaf, remarkably abundant root development and the ability of root differentiation on phytohormone-free medium. The presence of T-DNA and NPTII gene sequences in transformed black locust plants was shown by Southern analysis.

This dissertation is dedicated

to my parents,

Chang-Seek and Myeo-Yeon Han

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INTRODUCTION

Black locust (*Robinia pseudoacacia* L.), a nitrogen fixing and stress tolerant tree species, is planted globally. It has desirable wood characteristics, is fast growing, and many characteristics that facilitate its use as a research organism. The small genome size (Singh and Siminovitch, 1976), short generation interval (J. Hanover, personal communication), amenability to tissue culture (Chalupa, 1983; Davis and Keathley, 1985, 1987; Barghchi, 1987; Han and Keathley, 1988, 1991a, 1991b; and Merkle and Weicko, 1990) and receptability to *Agrobacterium* mediated genetic transformation (Davis and Keathley, 1990) make the species suitable as a model organism for genetic engineering of tree species.

Utilization. Black locust is an excellent source for the production of good dimension lumber, fenceposts, and poles (Keresztesi, 1988). The heartwood contains high concentrations (6% of dry weight; Smith et al., 1989) of flavonoids such as robinetin and robinin that provide decay resistance (Miller et al., 1987). Black locust fixes atmospheric nitrogen and thrives on poor sites; thus it is widely planted for land reclamation purpose. It provides quick stabilization of disturbed sites, enhances soil development by furnishing nitrogen and nutrient litter, fosters successional development of high-quality forest stands, and endows improved water quality (Ashby et al., 1985). As an animal feed source, black locust biomass is comparably nutritious to alfalfa in terms of crude protein content and is readily digestible since the lignin level is lower than alfalfa (Baertsche et al., 1986).

The high heat content, nearly twice that of *Populus* (Stringer and Carpenter, 1982), rapid juvenile growth, and prolific regrowth after harvest in short rotation intensive culture make the species ideal for biomass production (Miller et al., 1987) and/or multi-purpose plantations (Barrett et al., 1988). Black locust also has good characteristics for chemical pulping systems and is currently being used in the paper industry in Michigan (Miller et al., 1987). In addition, black locust has a great potential for beekeepers, as a large amount of quality honey may be gathered (Hayes, 1976; Keresztesi, 1988).

Limitation and potential problems. Although black locust is highly desirable in terms of wood quality, industrial utilization has been limited in North America by the locust stem borer (*Megacyllene robinae*) which tunnels into the bole in the larval stage. This ruins stem form, causes vulnerability to windthrow, and provides entry points for fungal pathogens such as *Fomes rimosus* (Hoffard and Anderson, 1982). Black locust is so competitive in mixed stands that the trees may overtop or damage other companion trees (Ashby et al., 1985; Chapman, 1935). In addition, the spines are hazardous to people and equipment.

Genetic improvement. Both sexual reproduction and vegetative multiplication must be used to achieve the maximum possible gain in a breeding program. Sexual hybridization transfers the genes which are already present in the breeding population, and vegetative propagation enables multiplication of genotypes that are new combination of genes. Typically, black locust breeding programs have been targeted for fast growth, stem straightness, frost resistance, increased nectar yield (Keresztesi, 1983), biomass production (Miller et al., 1987), and spinelessness (Kim, 1974). Such programs have great potential because heritability of these traits is high, allowing efficient selection for their improvement (Kennedy, 1983; Mebrahtu and Hanover, 1989). However, the application of breeding technology has been inefficient largely due to the difficulty of conducting controlled crosses (Tesfai Mebrahtu, personal communication). Consequently, the incorporation of biotechnology into black locust improvement programs will have a significant impacts on the attainment of specific breeding goals such as locust borer resistance or a spinelessness.

Tissue culture and genetic engineering. Tissue culture technology provides the means to exploit and capture the maximum genetic gain achieved in a tree improvement program in the shortest possible time (Hasnain and Cheliak, 1986). This is possible through a combined efforts of early selection of desirable characteristics and micropropagation of selected genotypes. Micropropagation systems for black locust have been developed using preformed shoot meristems (Barghchi, 1987; Chalupa, 1983, 1987; Davis and Keathley, 1987; Han and Keathley, 1991a,b), and leaf disks of young seedlings (Davis and Keathley, 1985). Other methods for basic research with black locust, include protoplast techniques (Han and Keathley, 1988) and somatic embryogenesis (Merkle and Weicko, 1990).

Although many *in vitro* techniques have been developed for black locust, no reliable method for the regeneration of whole plantlets from unorganized tissues has been developed. Such a regeneration system must be developed in order for genetic manipulation at the cellular and molecular levels to be utilized. Brown and Sommer (1982) reported regeneration of shoots from callus derived from black locust seedling shoot tips, but culture media and conditions were not described. The first two chapters of this dissertation describe experimental attempts to create an efficient regeneration system for black locust.

In forest tree species, the use of *in vitro* techniques has been confined mainly to tissues from juvenile sources (Sanchez and Vieitez, 1991). This limits the application of biotechnology as an alternative to traditional methods because trees are beyond their juvenile phase by the time they express most traits of interest.

While a reliable regeneration system is a prerequisite for genetic engineering of plant species, the introduction of foreign genes into a plant genome is a powerful approach for traits that are encoded by a single or few genes. *Agrobacterium*-mediated transformation has been an efficient way of introducing foreign genes into plant genomes (Klee et al., 1987).

Davis and Keathley (1989) transformed black locust with several strains of *Agrobacterium* spp. and detected stably incorporated T-DNA sequences in kanamycin resistant calli. However, transgenic black locust plants were not produced. The protocols for the transformation and regeneration of transgenic black locust were explored in the experiments which are described in this dissertation.

Objective. The studies conducted and reported herein were needed for the following reasons: 1) no method was available for shoot regeneration from unorganized tissues of black locust. The objectives addressed in the first chapter were to obtain shoot regeneration from callus tissues, to compare the responses of juvenile and mature explant sources, and to determine if superior response of a genotype to *in vitro* bud culture persisted in the shoots regenerated from callus; 2) there is a need to circumvent the problems related to the use of mature trees as a starting material in *in vitro* culture. In chapter 2, the ability to use tissues of mature black locust as starting material in a dedifferentiation-regeneration system was examined. Callus induction and subsequent shoot regeneration from the cambial tissues of mature black locust was demonstrated; 3) although the introduction of foreign genes into black locust was successful, no transgenic black locust plants have been reported. Chapter 3 reports the methods for *Agrobacterium rhizogenes*-mediated transformation and the recovery of transgenic black locust trees.

Conclusions and recommendations derived from the findings of these studies are presented at the end of this dissertation.

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CHAPTER 1

SHOOT REGENERATION FROM CALLUS-DERIVED FROM EXPLANTS OF JUVENILE AND MATURE *Robinia pseudoacacia* L.

ABSTRACT

Callus cultures were initiated from explants taken from two seedlings and two mature trees. Explant type had a significant effect on the frequency of primary callus production. Primary callus color was significantly affected by phytohormone level.

Regeneration of shoots from green calli occurred on MS medium containing 10 uM BAP alone or in combination with 1 uM NAA. The highest percentage of shoot regeneration was accomplished with primary callus induction on explants placed on MS medium with 1 uM NAA and 10 uM BAP, and shoot regeneration on MS medium containing 10 uM BAP, using seedlings of tree #2 as source of explants.

Differences in growth and rooting between shoots derived from callus of the two mature tree sources were apparent. Shoots from source M-1 produced a significantly higher number of shoots per explant than those of M-4. A significantly higher proportion of shoots regenerated from M-1 callus (57%) produced roots compared to shoots from M-4 calli (25%).

These results may reflect genotype specific differences among the explant sources.

INTRODUCTION

Black locust (*Robinia pseudoacacia* L.) is a leguminous tree species for which *in vitro* micropropagation systems have been developed. Chalupa (1983, 1987), Barghchi (1987) and Davis and Keathley (1987) all reported that rooted shoots were produced subsequent to inducing the elongation of preformed shoot meristems. The production of adventitious shoots from leaf disks has also been reported (Davis and Keathley, 1985). Such micropropagation procedures preserve the genetic integrity of propagules and thus allow clonal propagation of individual trees (Barghchi, 1987; Han and Keathley, 1991b) for use in basic research and breeding programs. Other tools for basic research of black locust, including protoplast techniques (Han and Keathley, 1988) and somatic embryogenesis (Merkle and Weicko, 1990) have been reported.

Along with the development of many *in vitro* techniques for black locust, *Agrobacterium*-mediated transformation has been reported for the species (Davis and Keathley, 1989). However, transgenic plants resulting from the genetic manipulation at the cellular and molecular level have not been achieved. In order for black locust to be utilized for such genetic manipulation, methods for regeneration of whole plantlets from callus tissues must be developed. Brown and Sommer (1982) reported regeneration of shoots from callus derived from black locust seedling shoot tips, but culture media and conditions were not described. In forest tree species, regeneration of whole trees from callus cultures initiated from mature individuals has been problematic (Bonga, 1982). Many protocols have been explored as a means of circumventing this problem, including preculture hedging of the explant source to rejuvenate the tissue.

One way that shoot regeneration frequency has been increased in some herbaceous species is by using genotypes which are superior performers *in vitro* as explant sources (Bingham et al., 1975; Koornneef *et al.*, 1986). Tree specific responses were observed among five mature black locust trees *in vitro* (Davis and Keathley, 1987).

To further investigate the use of germplasm screening to increase *in vitro* performance, two different mature trees, both of which appeared to respond well *in vitro* (Davis and Keathley, 1987), were evaluated for regeneration of shoots from subcultured callus. The first objective of this study was to obtain shoot regeneration from callus tissues. The second objective was to compare the response of these two mature trees to that of black locust seedlings. The third objective was to determine if superior response of a genotype (Tree #1) to *in vitro* bud culture (Davis and Keathley, 1987) persisted in the shoots regenerated from callus.

MATERIAL AND METHODS

Plant materials

Juvenile source: Two seedlots (designated J-2 and J-4) were collected from pods borne on mature trees #2 and #4, respectively as described in Davis and Keathley (1987). To produce aseptic seedlings, 40 seeds from each seedlot were boiled in water for 20 seconds, immersed in 5.25% NaOCl for 10-15 seconds, placed on sterile filter paper to dry, and to germinate in 18 x 150 mm culture tubes each containing 5 ml MS (Murashige and Skoog, 1962) basal medium with 0.5% (w/v) sucrose and 0.8% Bacto-agar (Difco). The seedlings were grown for 21 days in a growth chamber (50-75 uE/m^2/sec; 18 hr light/6 hr dark). Ten seedlings were chosen at random from each seedlot.

Mature source: Shoot cultures from trees #1 and #4 that were initiated during the study reported previously by Davis and Keathley (1987) were maintained for 9 months on MS medium supplemented with 0.32 uM BAP (6-benzylaminopurine) and 0.8% (w/v) Bacto-agar (Difco); referred to herein as shoot multiplication medium (SM). Cultures were initiated with shoot segments 2 cm or more in length. All cultures were incubated in a growth chamber under a 16 / 8 h light-dark photoperiod (50-75 uM/m^2/sec).

Callus initiation and growth

To induce callus, 20 sections, 2-3 mm in length, per treatment were taken from both hypocotyl and internodal segments of the shoot cultures of each clone and placed on callus initiation medium in 15 x 100 mm Petri dishes (10 explants per dish). The initiation medium was MS containing 5 or 10 uM BAP in combination with 1 or 5 uM NAA (naphthaleneacetic acid), using a 2 x 2 factorial design.

After 31 days, the number of explants that produced callus was counted, and green pieces of callus (5-7 mm in diameter) were excised from original explants and transferred to fresh callus medium of the same composition. Green calli were selected for subculture because past experiments indicated that morphogenesis did not occur from white callus tissues (Han and Davis, unpublished). After 25 days of further growth, green pieces of callus were removed from the callus mass and transferred to shoot regeneration media.

Shoot regeneration and proliferation

Shoot regeneration media, 10 ml in each 25 x 150 mm culture tube, was MS with BAP (10 uM) alone or in combination with NAA (1 uM). The number of calli that produced shoots was counted after 6 weeks of culture.

Shoots were excised from the callus tissue and transferred individually to 30 ml SM medium in each 55 x 130 mm culture jar to increase the number and standardize the size of propagules for further study. After 4 weeks, shoots greater than 2 cm in length were transferred to 10 ml of fresh medium in 25 x 150 mm culture tubes. Shoots less than 2 cm were not transferred, eliminating them from the experiment. Four weeks later, the number of shoots greater than 1 cm in length that were produced by each 2 cm long explant was counted, and shoots greater than 2 cm in length were again transferred to fresh SM medium. Shoots less than 2 cm in length were eliminated from the experiment. The number of shoots produced by each explant was again counted after 4 weeks of subculture.

Shoot cultures were subcultured for another 4 weeks of proliferation. This allowed elongation of the shoots for use in the rooting study. The shoots were cut to a length ranging from 2 to 2.5 cm and were used for the rooting test.

Root initiation

Shoots regenerated from callus were inserted individually in 10 ml of rooting medium, 0.1 strength MS supplemented with 1 uM IBA, in each 25 x 150 mm culture tubes. After

4 weeks, the number of shoots with at least one root was counted.

Plantlets were potted individually in a peat moss: perlite: vermiculite mix (1:1:1), watered with a fertilizer solution (1 g/l Peter's 20:20:20 in tap water), covered with plastic wrap to prevent desiccation, and placed in a growth chamber under a 16 / 8 h a light-dark regime. After 2 weeks the plastic was removed and the plantlets were fertilized weekly until transfer to a greenhouse.

Statistical analysis

The callus induction data were analyzed using a conventional analysis of variance for a factorial experiment (4 explant sources x 4 culture media). Percentage data were transformed using the arcsin transformation prior to the analysis (Steele and Torrie, 1980). To perform pairwise contrasts between clones, 2 x 2 contingency tables (*e.g.*, "rooted" vs. "non-rooted" for each clone) were constructed, and Chi-square values were calculated using the normal approximation (Steele and Torrie, 1980).

RESULTS

Callus growth was observed on induction media. Explant source had a significant effect on callus production (Table 1). Only 41% of the explants from tree M-1 produced callus;

whereas, an average of 97% of the seedling explants produced callus. In contrast, different phytohormone regimes in callus induction media did not significantly affect the number of explants which produced callus (F-test; $p>.7$).

However, phytohormone levels significantly affected callus color (Table 2). Mature explants produced green callus only from segments cultured on callus medium containing 5 uM NAA.

Regeneration of shoots from green calli occurred on medium containing 10 uM BAP alone or in combination with 1 uM NAA (Table 3). Since only green regions of the calli were transferred to the regeneration media, there was unequal representation of the callus induction treatments on the regeneration media. For example, since virtually all of the callus produced in the treatment with 1 uM NAA and 5 uM BAP was white (Table 2) and not growing vigorously, no calli from this treatment were transferred to regeneration medium. Likewise, callus produced from mature tissue cultured on initiated the medium containing 1 uM NAA and 10 uM BAP was white (Table 2), and hence was not used for regeneration attempt. Consequently, no statistical analysis of these data was attempted due to the lack of data.

Shoots were regenerated from callus produced by all four explant sources. Averaged across medium type, shoot regeneration from J-4 explants was poorest (1.1%), and optimum from J-2 explants was best (16.3%). The shoot regeneration (50%) was accomplished with callus initiated from J-2 explants on medium with 1 uM NAA and 10 uM BAP, and shoot regeneration on MS containing 10 uM BAP.

Shoots regenerated from J-4 callus failed to grow and develop. The average number of 5 per culture was produced from shoots derived solely from J-2 callus.

Differences in growth between shoots derived from callus of the two mature sources were apparent (Table 4). Shoots from M-1 multiplied rapidly after transfer to SM medium, yielding 324 shoots greater than 1 cm in length by

Table 1. Percent of explants derived from juvenile and mature black locust trees which produced callus after 32 days of culture on callus induction medium.

Explant	Explants callused[1] (%)
J-2	94.8 a
J-4	98.8 a
M-4	73.3 ab
M-1	41.0 bc

[1]Values followed by the same letter do not differ significantly (Tukey's test; alpha = .05).

Table 2. The effect of phytohormones in MS medium on color of calli from black locust explants after 32 days in culture.

Phytohormones (uM)	Callus color[1] Seedling	Mature	Average color[2]
NAA (1) BAP (5)	2.0	1.0	1.5 a
NAA (5) BAP (5)	6.0	4.0	5.0 ab
NAA (1) BAP (10)	5.5	1.0	3.25 ab
NAA (5) BAP (10)	5.5	5.5	5.5 b

[1]Callus color was determined subjectively on a scale of 1 (white calli) to 6 (green calli).

[2]Values followed by the same letter do not differ significantly (Tukey's test, alpha = .05).

Table 3. Observed differences in the proportion of calli grown from black locust explants which regenerated at least one shoot after culture on regeneration medium for six weeks.

Callus medium	Explant source	Shoot regeneration[1)] # explants / total		Avg. %
NAA(5) \	J-2	1/16	(6.3%)	
BAP(5) /	J-4	0/31		
	M-1	2/16	(12.5%)	
	M-4	0/32		
NAA(1) \	J-2	12/32	(37.5%)	
BAP(10)/	J-4	1/30	(3.3%)	
NAA(5) \	J-2	0/32		16.3
BAP(10)/	J-4	0/32		1.1
	M-1	2/32	(6.3%)	8.3
	M-4	2/32	(6.3%)	3.1

1) Value for the two regeneration media are averaged because the presence of 1 uM NAA in the medium had no effect on regeneration (pairwise contrast n.s., alpha = 0.05).

Table 4. Shoot proliferation characteristics of cultures derived from M-1 and M-4 explant sources.

Shoot growth interval[1]	Explant source	Number of shoots Start(>2cm)	End(>1cm)	Mean	± S.D.[2]
0 - 4	1	2	-	-	-[3]
	4	2	-	-	-
4 - 8	1	23	176	7.7	5.4
	4	3	14	4.7	0.6
8 - 12	1	47	324	6.9	3.4
	4	4	14	3.5	1.3

1) The number of weeks after excision of shoots from callus.
2) Mean number ± SE of shoots produced from a shoot.
3) - , data not collected

Table 5. Percent rooting of shoots regenerated from J-2, M-1, and M-4 callus cultures.

Time (weeks)	Explant source	Rooting[1] (%)	Mean
4	J - 2	9/46 (19)	1.6
	M - 1	40/72 (56)	1.2
	M - 4	3/16 (19)	1.3
5	J - 2	9/46 (19)	1.6
	M - 1	40/72 (56)	1.4
	M - 4	4/16 (25)	1.3
6	J - 2	11/46 (24)	1.9
	M - 1	41/72 (57)	1.6
	M - 4	4/16 (25)	1.5

1) The number of explant which produced at least one root is shown in the numerator, divided by the total number of explants.

twelve weeks after excision of the shoots from callus. In contrast, shoots from M-4 multiplied slowly; only 14 shoots longer than 1 cm were obtained at the end of the same period.

A significantly higher proportion of shoots from M-1 callus rooted (57%) compared to both J-2 (24%) and M-4 (25%) calli (Table 5; pairwise difference at alpha = 0.05).

Discussion

Explant source had a significant effect on callus induction. Almost all of the hypocotyls from the juvenile *tissues produced callus (97%), whereas only 57% of the mature explants produced callus. However, callus induction from M-4 explants did not differ significantly from either of the juvenile explants, but the response of M-1 explants did. This indicates the callus induction response was dependent on genotype, not simply on maturity. Similarly, we found a significant genotypic effect on cambial tissue cultures derived from mature black locust (Han and Keathley, 1991a)

It might be expected that the juvenile explants would exhibit more variability in response on the callus induction media, since the half-sib seed lots were genetically heterogeneous, in contrast to the shoot cultures that were of clonal origin. This was not observed, and may indicate that hypocotyl explants, due to either anatomical or physiological differences from stems, form callus more readily. Alternatively, this may reflect a difference between juvenile and mature tissue. These contentions could be tested if explants of shoot cultures derived from individual seedlings were compared to mature proliferating shoot cultures. The differential responses of callus induction between juvenile and mature tissues have been observed on cambial tissues isolated from epicormic branches (known as "in juvenile phase"; Bonga, 1982) that produced a higher frequency of callus induction than those from crown branches (known as in mature phase) (Han and Keathley, 1991c).

Both M-1 and M-4 explants produced sporadic callus on MS medium containing 1 uM NAA, regardless of the BAP level. This was not the case with the juvenile sources, which produced green calli on medium containing 1 uM NAA and 10 uM BAP. This implies that sustained growth of callus from mature black locust tissues may require these higher auxin levels, although it is also possible that this requirement is specific to these two mature genotypes.

Regeneration of shoots occurred from callus of both mature explant sources. Although the efficiency of shoot regeneration from the mature explant sources was rather low under these conditions (less than 10%), these experiments demonstrated the capacity of calli derived from mature tissues of this species to regenerate *in vitro*. Previously, shoot regeneration from callus of black locust had been demonstrated only in seedling tissues (Brown and Sommer, 1982).

We expected that the calli derived from juvenile explants would regenerate shoots at a higher frequency than that of mature origin. This was the case for calli of the J-2 source.

It is interesting, however, to note that M-1 and M-4 calli had a higher shoot regeneration (8.3% and 3.1%, respectively) than J-4 calli which had a shoot regeneration percentage of 1.1%. As was indicated by the callus induction data, these results suggest that the juvenility or maturity of an explant source *per se* is not necessarily a reliable predictor of organogenesis, and should not always be the primary consideration in efforts to develop more efficient regeneration systems for forest tree species.

The most efficient shoot regeneration occurred from J-2 calli derived from MS induction medium containing 1 uM NAA and 10 uM BAP. For these calli, the transfer to regeneration medium was essentially a subculture step, where both callus and shoots proliferated throughout the experiment (even though unorganized callus tissues were selected for transfer at each transfer step). This shoot proliferation was not observed with J-4 calli, calli from hypocotyls of a locally collected, bulked seedlot (Han, unpublished), or calli derived from the mature explants. The difference between the J-2 and J-4 calli could not be attributed to noticeable differences in seedling vigor (personal observation), or average seed weight (data not shown). If this characteristic is common among individuals from the J-2 seedlot, these seedlings may be useful as explant sources for increasing the efficiency of shoot regeneration from protoplast-derived calli of black locust.

Shoot multiplication occurred more readily from the M-1 than the M-4 derived calli. This is reflected in both the relative and the absolute increases in shoot number that occurred on SM medium. Eight and 12 weeks after the excision of shoots from callus, the shoots from the M-1 source produced a 7.7- and 6.9-fold increase in shoot number, while shoots from the M-4 source produced a 4.7- and 3.5-fold increase, respectively.

Even more pronounced than the relative increases in shoot production were the clonal differences for the total number of shoots produced. By the end of the third interval, the M-1 source had produced 23-fold more shoots than M-4. When dormant bud explants were taken from these trees and compared under similar culture conditions, shoot proliferation from M-1 was also found to be consistently superior to that of M-4 source (Davis and Keathley, 1987).

Shoots from M-1 were also more likely to root than the M-4 source. This difference between the clones, originally reported in the previous study using bud cultures (Davis and Keathley, 1987), was highly significant and was observed on medium containing IBA, NAA, or a combination of these 2 phytohormones. The shoots regenerated from callus reflected the same clonal differences in rooting on MS containing IBA. Rooting of shoots regenerated from callus was less than in shoots derived from bud culture (Han and Keathley, 1991c). This difference in rooting efficiency may be due to physiological differences in tissue or to environmental differences such as container size or medium volume. The lower rooting percentage was obtained with shoots produced from callus of juvenile source (J-2) compared to shoots from mature source (M-1). Since juvenile tissues are known to have better rooting ability than mature ones (Sanchez and Vietez, 1991; Hackett, 1987), it is notable that the mature sources produced more roots than juvenile explants. The difference may indicate the genotypic effect of tree #1 rather than physiological differences in tissue.

The rooting and shoot proliferation data suggest that the genetic or epigenetic factors responsible for the differential performance of the two mature sources (Davis and Keathley, 1987) persisted through the somatic cell dedifferentiation and regeneration processes associated with callus growth. Performance in tissue culture has been related to genotype differences (Reisch and Bingham 1980, Komatsuda and Ohyama 1988). It has been shown previously that explant pretreatments or phytohormones can completely or partially override differential responses due to genotype (Gharyal and Maheshwari 1983, Komatsuda and Ohyama 1988). Our data indicate that different phytohormones in the regeneration of shoots from callus of black locust did not override clonal differences.

Although the two mature sources are certainly distinct genotypes, either physiological or genetic factors could be responsible for the differences we observed in the *in vitro* performance of the two clones. Since the shoot cultures were originally derived from trees of flowering age, crosses could be performed to test such hypotheses. Further studies have allowed the development of more efficient callus growth and shoot regeneration procedures for this genotype (Han and Keathley, 1991a). We hope that shoot cultures of this clone (Han and Keathley, 1991b) can provide a genetically and physiologically uniform explant source for studies aimed at both the regeneration of shoots from protoplasts (Han and Keathley, 1987) and genetic transformation of black locust.

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CHAPTER 2

CHARACTERISTICS OF CAMBIUM-DERIVED CALLUS CULTURES AND REGENERATED SHOOTS IN MATURE BLACK LOCUST (*Robinia pseudoacacia* L.).[1)]

ABSTRACT

Primary callus cultures were derived from cambial tissue of mature black locust placed on MS medium containing 5 uM BAP and 10 uM NAA. Genotype had significant effects on callus growth and subsequent shoot regeneration. Diameter growth of callus was not affected by auxins or light intensities; however, the color and texture of callus was significantly influenced by genotype, phytohormone, light intensities, and their interactions. Eight month storage of cambial tissue at 4 ^{o}C resulted in significant changes in diameter growth but did not affect callus color or medium browning. Shoot regeneration was highly tree specific.

INTRODUCTION

In vitro micropropagation of black locust (*Robinia pseudoacacia* L.) has been reported from auxiliary buds (Chalupa 1983; Davis and Keathley 1987; Barghchi 1987) and from leaf disks (Davis and Keathley 1985). Along with these techniques, isolation and culture of protoplasts (Han and Keathley 1988) and regeneration of black locust via somatic embryogenesis (Merkle and Wiecko, 1989) have extended current *in vitro* methods for this species. Additionally, Davis and Keathley (1989) were able to transform black locust hypocotyls with several strains of *Agrobacterium* and to detect stably incorporated T-DNA sequences in calli resistant to kanamycin.

Manipulating plants genetically at the cellular and molecular levels for use in plant improvement programs requires the ability to regenerate whole plants from unorganized tissues. In black locust, successful shoot regeneration has been reported from seedling-derived callus and callus derived from shoot cultures of mature trees. Significant tree specific responses were observed in cultures from both half-sib seedling and mature origins.

We report a protocol for callus initiation and culture from the cambial tissue of mature black locust trees, delineates methods for obtaining subsequent shoot regeneration from the callus, and discuss persistent tree specific response to *in vitro* manipulation.

MATERIALS AND METHODS

Explant preparation: Branches (0.5 to 1.0 cm in diameter) were removed from 4 mature trees (23-26 years-old) in March of 1988. The branches were cut into 3-4 cm pieces and washed with full strength of detergent (Liqui-nox) and placed under running tap water for 1 hr, immersed in 70% ethanol for 1-2 min and soaked in a 50% Clorox solution for 10-15 min followed by 5 washes with sterile distilled water. The branch bark was removed and a cambium layer 0.2-0.5 mm thick and 3 x 4 mm was removed out using a scalpel.

Explant culture: Explants of cambial tissue were placed in Petri dishes (15 x 200 mm) each containing 25 ml of MS (Murashige and Skoog, 1962) basal medium supplemented with various phytohormones. Number of explants started and phytohormone levels are given in the Tables for each experiment. MS basal medium, 0.8% Sigma-agar, and 2% sucrose were used throughout and adjusted to pH 5.8 autoclaving for 15-20 min at 120^{o} C.

Experiments were conducted in a controlled environment chamber at 25^{o} C, with an 18 hr light/6 hr dark lighting regime (50 uE/m^2/sec). Callus was maintained by subculturing at 4 week intervals. Callus growth was scored by measuring diameter, color, browning of medium and texture of the callus. Color, texture, and medium browning were scored in the following manner; color: brown or dead=0, white=1, whitish green=2, greenish white=3, green=4; texture: very friable=1, friable=2, firm=3, very firm=4; medium browning: none=1, light=2, moderate=3, dark=4.

Shoot regeneration and rooting: Callus cultures that have been subcultured at least 5 months were used for regeneration experiments. Five calli per Petri dish were transferred to MS medium containing various concentrations of BAP alone or in combination with NAA (Table 5). The number of shoots regenerated and callus growth were scored at 13 weeks. Shoots (1 cm in length) were excised from the callus tissue and transferred individually to 10 ml of shoot multiplication medium (MS + 0.5 uM BAP) in each 25 x 200 mm test tube. The proliferating shoots (2 cm in length) were transferred to rooting medium (1/10 strength MS supplemented with 1 uM IBA and no sucrose). Rooted plantlets were potted in a container using peat moss, pearlite, and vermiculite (1:1:1), watered with Peter's 20:20:20 (1 g/l), and covered with plastic wrap.

Statistical analysis: The data for callus initiation and growth were analyzed separately for each treatment using a general linear model analysis of variance/covariance (GLM-ANOVA; Hintze 1987). Correlation coefficients among the diameter, color, texture of callus, and browning of medium were calculated as Spearman's rank correlation (Steele and Torrie, 1980). The data for each treatment were separated by Duncan's New Multiple-Range Test (Steele and Torrie, 1980) where the GLM-ANOVA indicated significance.

RESULTS

Analysis of the data obtained from preliminary experiments with hypocotyl indicated that no significant difference (alpha = .05) in callus growth existed between cultures initiated on MS medium containing 2,4-D or NAA (data not included). Both cytokinin type and cytokinin/auxin interaction had significant effects on diameter growth and callus induction. The inclusion of BAP in the MS medium resulted in significantly greater callus diameter growth, higher callus induction frequency, and greater shoot regeneration (data not included). Based on these results, BAP and NAA were selected for further study.

To identify optimal concentrations of the phytohormones, four different concentrations each of BAP and NAA were tested (Table 1) using cambial tissues from tree #3, which gave an intermediate response in preliminary experiments (data not presented). Analysis of variance showed

Table 1. Effect of phytohormone concentration on cambium callus initiation.

Phytohormones NAA	BAP (uM)	Explants (No.)	Average Diameter (mm)	Color (x)	Callus %
0	0	20	no callus	dead	0
0	1	20	5.8b[1)]	1.1abc	95
0	5	20	6.8bcd	1.2bcd	100
0	25	19	5.6b	0.4a	84
1	0	10	not scored	dead	10
1	1	17	9.4e	1.8cde	100
1	5	20	8.9de	2.2ef	100
1	25	19	5.4b	0.8ab	100
10	0	19	6.18bc	1.2bc	73
10	1	15	9.2e	2.0ef	100
10	5	20	12.0f	2.2ef	100
10	25	18	8.2cde	2.1ef	66
50	0	10	12.7f	1.0ab	90
50	1	20	9.1e	1.9def	100
50	5	20	8.7de	2.6fg	95
50	25	14	3.0a	3.0g	78

1) Values followed by different letters are significantly different by Duncan's NMR test (alpha = 0.05).

that BAP, NAA level and their interaction were highly significant for the diameter of induced callus (F-test; alpha = 0.01). Only the BAP main effect and the interaction term were significant for callus color (F-test; alpha = 0.05 and 0.01, respectively). On the basis of the diameter and callus color data, MS augmented with 10uM NAA and 5uM BAP was chosen as the black locust callus induction medium.

Tree specific responses in cambial callus growth were observed for both freshly collected explants and explants from tissues that had been stored for eight months (Table 2). Analysis of variance showed that individual trees differed significantly for callus diameter growth, callus color, and browning of medium (F-test; alpha=0.01). The interaction between genotype and initiation time was highly significant for callus diameter growth and browning (F-test; alpha=0.01). Storage of cambial material did not significantly affect callus color or medium browning, but did result in significant changes in callus diameter growth (F-test; alpha=0.05).

Callus diameter was correlated with degree of green color of callus and media browning (Spearman's rho = -0.43 and -0.69, significant at 1 % level; respectively). Callus color and media browning were not significantly correlated (Spearman's rho = 0.13, sample size of 88). Overall, tree #4 produced the most rapidly growing callus.

To test the effect of auxin and light intensities on callus maintenance, two auxins, NAA and 2,4-D, and two light intensities (51 and 8 $uE/m^2/sec$) were applied in a factorial design (Table 3). Different auxins and light intensities did not have significant effects on callus diameter growth. Explant source in combination with auxin was significant (Table 4). The callus color was significantly affected by tree #, tree # by auxin interaction, and light intensity (Table 4). All main effects were significant for callus

Table 2. The effect of 8 months storage at 4 °C of branches collected in March on cambium-derived callus initiation.

Tree No	Culture initiation[1)]	Callus Diameter(mm)	Color (x)	Browning (x)
1	April (10)	10.8+.6c	2.2+.3b	3.2+.2f[2)]
	November (12)	7.6+.6b	3.0+.2c	3.5+.2g
2	April (10)	10.6+.6c	3.4+.3c	1.4+.2a
	November (11)	15.9+.6e	2.1+.2b	1.3+.2a
3	April (10)	11.7+.6c	3.3+.3c	2.2+.2c
	November (9)	4.8+.7a	3.0+.2c	3.7+.2g
4	April (10)	12.9+.6d	2.5+.3b	2.5+.2d
	November (16)	14.3+.5e	1.2+.2a	1.3+.2a

1) Number in parenthesis = number of explants.
2) Values with different letters are significantly different by Duncan's NMR test (alpha = 0.05).

texture (Table 4). No significant correlation was found between callus diameter growth and firmness of callus (Spearman's rho = -0.06; sample size of 320) on callus medium. Greenness and diameter growth were not correlated (Spearman's rho = 0.09, sample size of 320), however, the greener callus tended to be firmer in texture (Spearman's rho = 0.24, significant at 1% level). Both the type of auxin and tree from which the explants were taken had significant effects on callus diameter growth and greenness of the callus following transfer to shoot regeneration medium (Table 6). When NAA was added to the MS medium, callus diameter growth and the greenness of callus rapidly increased (Table 6).

The only combination that resulted in shoot regeneration was when callus from tree #1 was transferred to MS medium that lacked NAA (Table 5). Storage of explant tissue for eight months did not significantly alter shoot regeneration (Pairwise T-test; t = 0.89, df = 413).

Shoots were increased on shoot multiplication medium. The number of shoots greater than 1 cm in length averaged 4.75 per shoot culture tube. Twenty-five percent of the shoots rooted (Figure 4) and were subsequently potted and transferred to the greenhouse (Figure 5).

Table 3. Increase in cambium callus diameter growth and change in color/texture with different auxins and light intensities during a 4 week interval.

Trees No	Medium[1]	Light[2]	Increased in diameter(mm)	Change in color	Change in texture
1	MSN	Full	13.7de	-0.9de	0.0cd[3]
		Dim	10.9ab	-1.8ab	-0.5b
	MS2	Full	12.1bcd	-1.7bc	-0.9a
		Dim	11.3abc	-1.8abc	-1.0a
2	MSN	Full	9.9a	-1.9ab	0.6f
		Dim	10.1a	-2.1a	0.0cd
	MS2	Full	10.9ab	-1.5bc	0.7f
		Dim	11.0ab	-1.3cd	0.4ef
3	MSN	Full	13.9e	-0.2f	0.2de
		Dim	14.2e	-1.7bc	-0.4b
	MS2	Full	13.4de	0.6ef	-0.2bc
		Dim	14.4e	-1.0de	-0.3bc
4	MSN	Full	12.7cde	-0.3f	-0.2bc
		Dim	13.3de	-1.0de	-0.3bc
	MS2	Full	12.3bcd	-0.8de	-0.3bc
		Dim	10.7ab	-0.4ef	-0.1bcd
	SE ($\pm$)		0.52	0.20	0.12

1) Medium: MSN = MS + 5 uM BAP + 10 uM NAA; MS2 = MS + 5 uM BAP + 10 uM 2,4-D.
2) Light : Full = 51 uE/m2/sec; Dim = 8 uE/m2/sec.
3) Values followed by different letters are significantly different by Duncan's NMR test at alpha=0.05.
4) Each treatment has 20 explants.

Table 4. Analysis of variance (GLM-ANOVA) for cambium-derived callus growth, color, and texture with different auxin and light intensities.

Source	df	Change in diameter	Change in color	Change in texture
Tree	3	162.0**	30.0**	13.0**
Auxin	1	8.128	1.25	1.38*
Tree x auxin	3	21.03**	4.34**	3.04**
Light	1	10.87	26.5**	4.75**
Tree x light	3	21.85**	8.04**	0.84*
Auxin x light	1	0.253	12.8**	2.63**
Tree x auxin x light	3	14.57*	2.06*	0.10
Error	304	5.394	0.79	0.28

* Mean square significant at the 0.05 significance probability.
** Mean square significant at the 0.01 significance probability.

Table 5. Shoot regeneration from cambium-derived callus of mature black locust after culture for 13 weeks.

Trees No	Medium NAA	BAP(uM)	Explants No[1]	% explants produced shoot Exp.I	Exp.II	Diameter change	Color change
1	0	10	12	0.0[2]	8.32[3]	2.7	-3.3[4]
	0	25	18	27.8	33.3	7.8	-2.6
	0	50	12	0.0	16.7	3.4	-3.4
	10	10	18	0.0	0.0	15.4	-0.2
	10	25	18	0.0	0.0	15.1	-0.3
	10	50	18	0.0	0.0	14.3	-0.2
2	0	10	18	0.0	0.0	2.3	-1.3
	0	25	18	0.0	0.0	0.1	-1.4
	0	50	18	0.0	0.0	0.4	-1.2
	10	10	18	0.0	0.0	14.2	-0.2
	10	25	18	0.0	0.0	13.3	-0.2
	10	50	12	0.0	0.0	12.5	0.2
3	0	10	18	0.0	0.0	10.0	-3.0
	0	25	18	0.0	0.0	3.4	-3.4
	0	50	18	0.0	0.0	3.1	-3.6
	10	10	18	0.0	0.0	17.0	0.0
	10	25	18	0.0	0.0	16.2	-0.3
	10	50	18	0.0	0.0	14.7	-0.9
4	0	10	18	0.0	0.0	15.1	-1.1
	0	25	18	0.0	0.0	12.6	-1.7
	0	50	18	0.0	0.0	11.2	-1.4
	10	10	18	0.0	0.0	17.7	-0.4
	10	25	18	0.0	0.0	18.2	-0.1
	10	50	18	0.0	0.0	17.9	-0.1
	Total mean		414	1.2	2.4	10.8	-1.3

1) No. of explants for Experiment 2.
2) Experiment 1: For each treatment, twenty calli from fresh cambial tissues were used for regeneration test.
3) Experiment 2: Calli from cambial tissues stored for eight months were used for regeneration test.
4) Data for diameter and color were taken from Experiment 2.

Table 6. Analysis of variance (GLM-ANOVA) for increase in callus diameter growth with different BAP levels on the data from Table 5.

Source	df	Increase in diameter	Change in callus color
Tree	3	1310.8**	31.33**
NAA	1	5085.0**	245.9**
Tree x NAA	3	298.59**	21.45**
BAP	2	114.07**	0.566
Tree x BAP	6	51.409*	1.898
NAA x BAP	2	17.068	0.254
Tree x NAA x BAP	6	51.979*	0.976
Error	390	23.960	1.165

* Mean square significant at the 0.05 significance probability.

** Mean square significant at the 0.01 significance probability.

DISCUSSION

The optimum phytohormone levels for the initiation of callus from mature cambial tissue were found to be 5 uM BAP and 10 uM NAA. This combination produced firm primary callus that was competent for shoot regeneration. The frequency of callus induction from mature cambial tissues (90-100 %) was similar to that of juvenile tissues, indicating that unlike organogenesis, cellular growth *in vitro* is not constrained by explant age. Explant source (tree) is the significant factor in determining callus diameter growth, but light intensity and auxin type become important if we look at the other indicators of metabolic activity, such as the color and texture of the callus. Since the induction of firm, green callus has been a prerequisite for regeneration in black locust, these studies showed that culture conditions, as well as explant source, must be considered in identifying an optimal culture condition. Furthermore, significant interactions between the source of explants and environmental factors indicated that not only are both significant, but different trees respond to change in light and auxin levels differently. Differential responses in photosynthetic activity have also been observed among half-sib families of black locust when exposed to different environmental regimes *in vivo* (Mebrahtu and Hanover, 1990). While the expression of these metabolic and physiological differences *in vitro* makes it difficult to develop a culture system, it also provides a promising avenue to pursue the mechanisms that underlie them.

Callus color and firmness were positively correlated, with the firmer callus greener in color. Diameter growth of callus was not significantly correlated with firmness or greenness of the callus at low cytokinin concentrations. However, in the treatments with high cytokinin levels, the correlation between diameter growth and greenness was highly significant (Spearman's rho = 0.67, significant at 1 % level). The greener color and firmness of callus grown on medium containing high cytokinin concentrations may result from cytokinin-derived plastid development (Partheir, 1979). It has been hypothesized that cell wall loosening triggered by auxin may be due to either the activation of wall loosening factors, such as protons (Cleland, 1987), or increased extracellular cellulase activity (Fry, 1989). Auxin/cytokinin balancing effects have been observed in plant tissue culture (Krikorian, et al., 1987), but the mechanism responsible for this phenomenon has not been identified.

When callus was placed on MS media containing high BAP but lacking NAA rather than callus medium (10 uM NAA and 5 uM BAP), diameter growth and greenness were significantly lowered, confirming the importance of maintaining the auxin/cytokinin balance in *in vitro* culture systems. Medium browning in tissue culture is generally thought to be a result of the oxidation of phenolic compounds by polyphenoloxidase (Hu and Wang, 1983). In these studies medium browning was positively correlated with the greenness of callus. Since the degree of medium browning varied among genotypes, with the highest degree of browning in tree #1, and since tree #1 has shown consistently better performance, the relationship between the degree of medium browning and shoot differentiation is of interest for further study. The differential *in vitro* growth response has also been observed in bud cultures of the same trees (Davis and Keathley, 1987), shoot regeneration from hypocotyl-derived callus of black locust, and shoots regenerated from callus derived from internodal segments of *in vitro* shoot cultures of mature black locust (chapter 1). Komatsuda et al. (1989) conducted a complete diallel set of crosses to elucidate the genetic basis of the genotypic differences in callus culture and subsequent shoot regeneration in barley. From their result, significant dominant gene actions with high heritability as well as additive gene effects were found in *in vitro* traits. Considering their findings, our results when combined with previous works with these trees suggest that the superior *in vitro* response of tree #1 is likely due to true genetic rather than epigenetic effects. Several observation support this contention. First, the differential response observed in mature bud culture (Davis and Keathley, 1987) has been persistent through callus-plant regeneration (Han et al., 1990). Second, the studies reported here indicate that the major controlling factor in mature cambium culture is associated with the tree, rather than the culture environment. Finally, Komatsuda et al. (1989) confirmed that the expression of callus growth and shoot differentiation are governed by different genetic mechanisms, and our results also show a differential response where tree #4 was best for callus culture; whereas, tree #1 gave optimum shoot regeneration.

The use of cambial tissues provide advantages over other tissues from mature trees due to its year-round availability and relatively high chance of avoiding systematic contamination problems caused by soil-born bacteria. Furthermore, cambial explants offer the possibility of immediate gene transfer without waiting through an initial culture induction period, as demonstrated in ongoing experiments in which cambial tissues of black locust have produced kanamycin resistant calli when co-cultivated with an *Agrobacterium* strain which has a kanamycin resistance gene inserted into a disarmed Ti plasmid (Han and Keathley, unpublished). The ability to store cambial tissue facilitates testing and the development of tissue culture systems by simplifying the logistics of the process. Cambial tissues could also be preserved in tissue banks for conservation of tree germplasm by either cryopreservation or cold-room storage methods.

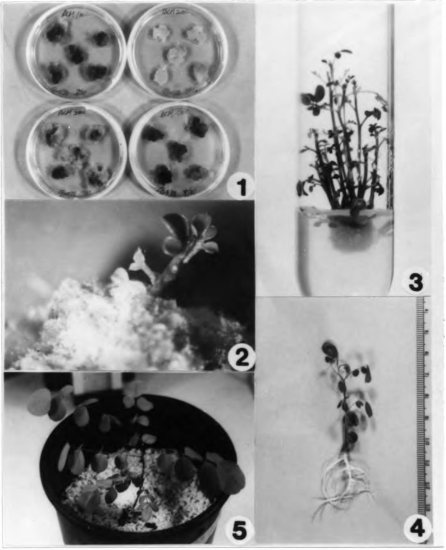

Figure 1: Cultures of cambium-derived callus from four mature black locust (Robinia pseudoacacia L.).
Figure 2: Shoot organogenesis from callus of tree #1 on the regeneration medium (bar = 3 mm).
Figure 3: Multiplication of the regenerated shoots on MS + 0.5 uM BAP.
Figure 4: Subsequent rooting of the multiplied shoots.
Figure 5: Potted black locust which regenerated from cambial tissue of mature tree (pot diameter = 25 cm).

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CHAPTER 3

Transformation of *Robinia pseudoacacia* L. *via* a hypocotyl-*Agrobacterium rhizogenes* system.

ABSTRACT

Hypocotyl segments inoculated with *Agrobacterium rhizogenes* strains R1500 and R1601 produced hairy roots when cultured on MS medium without phytohormone. Ri-induced hairy roots were faster growing and larger than normal roots. They also had a marked development of lateral roots when cultured on black locust callus culture medium (BCM) supplemented with 100 ug/ml kanamycin. Less than 5% of adventitious roots spontaneously produced shoots. The regenerated shoots were multiplied and rooted in the presence of 100 ug/ml kanamycin. Leaf pieces from the shoot cultures were tested for the expression of a kanamycin resistance gene by culturing on BCM with 100 ug/ml kanamycin. All leaf pieces taken from transformants induced callus on BCM with 100 ug/ml kanamycin while leaf pieces from normal plants died. The hairy root phenotype which has been characterized as having wrinkled leaves and abundant root development was evident after 3 months of shoot development in pot from potted transformants. Southern analysis showed the presence of both T_L-DNA and NPTII gene sequences.

INTRODUCTION

The infection of susceptible plants with virulent strains of *Agrobacterium tumefacience* or *A. rhizogenes* results in the formation crown gall tumors or "hairy root" disease (for review, see Bevan and Chilton, 1982; Nester et al., 1984; Zambryski, 1988; Zambryski et al., 1989). These pathogenic responses result from the transfer and expression of the transfer DNA (T-DNA) of the Ti- or Ri-plasmid in the plant genome (Binns and Thomashow, 1988; Klee et al., 1987). Endogenous T-DNA directed synthesis of phytohormones, both auxin (Inze et al., 1984; Schroder, 1984) and cytokinin (Akiyoshi et al., 1984; Barry et al., 1984), forms the basis for phytohormone autotrophic growth of the transformed cells. Intact T-DNA also encodes genes which direct the synthesis of different opines, and these unique amino acid derivatives can be utilized by the bacterium as its sole carbon and nitrogen source (for review, see Tempe et al., 1984).

Although the tumor-inducing (Ti) plasmids of *A. tumefaciens* have been used more frequently as a vector for plant genetic transformation than the root-inducing (Ri) plasmids of *A. rhizogenes*, in recent years vector systems based on the Ri-plasmid have been developed and used for gene transfer (for reviews, see Birot et al., 1987; Petersen et al., 1989; Tepfer and Casse-Delbart, 1987). The advantage of Ri- compared to Ti-plasmid mediated transformation is that the presence of the exogenous Ri T-DNA segment does not prevent the regeneration of whole fertile plants from hairy roots. Intact plants of various plant species have been obtained (Ackermann, 1977; Spano and Costino, 1982; Tepfer, 1984). The *A. rhizogenes* agropine-type strain A4 transfers two distinct DNA segments, left (T_L) and right (T_R), of T-DNA into plant cells (White *et al.*, 1985). The virulence of the *A. rhizogenes* A4 strain was significantly increased by using the *vir* region of a "supervirulent" plasmid pTiBo542 in *trans* (Pythoud et al., 1987).

Long generation cycles, space requirements for large segregating populations, the large size of trees, and the lack of genetically pure lines are all examples of limitations that may be breached with the ability to transform a particular tree species. Even though *Agrobacterium* can infect a wide spectrum of woody plants, including many angiosperms and some gymnosperms, successful application of an *Agrobacterium*-mediated transformation system has been limited, partially because the system for the regeneration of whole plants from transformed cells has not been developed (Hassig et al., 1987). Transformation of woody species and subsequent regeneration of transgenic plants has been reported for only a few genera including *Populus* (Fillatti et al., 1987; Pythoud et al., 1987; McCown et al., 1991), *Juglans* (McGranahan et al., 1988), *Malus* (James et al., 1989), and *Vitis* (Mullins et al., 1990).

A number of characteristics make black locust ideal for transformation studies (see 'Introduction' of this dissertation). Davis and Keathley (1989) were able to transform black locust with several different strains of *Agrobacterium* and to obtain kanamycin-resistant calli. However, no transgenic black locust plant was regenerated. The objective of this study was to obtain transgenic black locust plant through a *Agrobacterium*-mediated transformation.

MATERIALS AND METHODS

Bacterial strains. *Agrobacterium* was grown on Luria Bertani (LB) medium supplemented with appropriate antibiotics when necessary. The concentrations of antibiotics used were 100 ug kanamycin (Sigma Chemical Company) per ml and 100 ug ampicillin (Sigma Chemical Company) per ml. *A. rhizogenes* R1500 and R1601 (Pythoud et al., 1987) were kindly provided by J.M. Davis from M.P. Gordon's laboratory.

Tissue culture systems and infection procedure. *In vitro* culture systems for transformation studies have been developed through the experiments described in chapters 1 and 2. The small segments (0.5 to 1.0 cm in length) of hypocotyl were soaked for several minutes in an overnight culture of *Agrobacterium rhizogenes*, and placed on agar (0.6%) solidified Murashige and Skoog (MS) medium (1962) without phytohormones (MSO). After 3 days, the explants were transferred to MSO containing 300 ug/ml cefotaxime (Sigma) and 300 ug/ml vancomycin (Sigma) to kill the bacteria. The tissue was subsequently maintained on MSO supplemented with 500 ug/ml of both antibiotics.

Selective culture. Induced hairy roots (> 1 cm in length) were excised and cultured on MS medium containing 10 uM NAA, 5 uM BAP, and 100 ug/ml kanamycin to induce callus and shoot regeneration. Transgenic callus was maintained on the same induction medium. Shoots that regenerated from the hairy roots were inserted in MS medium supplemented with 0.6% agar, 2% sucrose, 1 uM BAP (BSM), and 100 ug/ml kanamycin. Rooted putative transgenic black locust plant lets were grown in a peat moss: perlite: vermiculite mix according to Han et al. (1990).

To test for the expression of kanamycin resistance in the transgenic plants; the following procedures were used: 1) both transformed and normal shoots were transferred to rooting medium (BRM; Han and Keathley, 1991) in the presence of 100 ug/ml kanamycin (Sigma) and survival and rooting ability was evaluated after 4 weeks of culture. 2) leaf pieces from both transformed and normal individuals were cultured on callus induction medium (BCM; chapter 2) supplemented with 100 ug/ml kanamycin (Sigma). Explants were evaluated for survival and callus induction.

Plant DNA isolation. Total plant DNA was isolated efficiently as in Draper and Scott (1988). Young leaf material was harvested from the potted plants, frozen in liquid nitrogen and ground to a fine powder using a pre-chilled mortar and pestle. The powder was then thawed in extraction buffer (15 ml/g fresh weight; 2.0M Tris-HCl pH 8.0, 0.5M EDTA pH 8.0, 5.0M NaCl, 10 mM 2-mercaptoethanol), and 1/7.5 volume of 10% SDS was added. After mixing thoroughly by gentle shaking the suspension was incubated for at least 20 min. in a 65 ^{o}C waterbath. 5M potassium acetate (1/3 volume of extraction buffer) was then added and mixed by repeated inversions and followed by a 30 min. incubation on ice. The protein/SDS precipitate was removed by centrifugation at 12,000 x g for 30 min (4 ^{o}C) in a HB4 rotor. The supernatant was filtered through 4 layers of Miracloth^{r} into a clean tube and DNA was precipitated by adding pre-chilled isopropanol (-20 ^{o}C; 2/3 volume of total solution). The precipitated DNA was hooked out using a 1.5 ml Pasteur pipette, transferred to precipitation buffer in a microfuge tube (1 ml/g fresh weight; 76% ethanol, 10mM ammonium acetate), and set for 20 min at room temperature.

The DNA was pelleted by centrifugation for 30-60 seconds in an Eppendorf microcentrifuge. The pellet was dissolved in the smallest possible volume of Tris-EDTA buffer (pH 8.0).

Southern analysis. DNA was digested with restriction enzyme HindIII under the conditions specified by the manufacturer (Boehinger Mannheim). The digested DNA was size fractioned by electrophoresis in 0.7% agarose gel with TBE buffer (Sambrook et al., 1987) and then transferred to Zeta-probe blotting membrane by DNA alkaline blotting as specified by the manufacturer (Bio-Rad Laboratory).

Two probes were used. The first, a purified PstI fragment, which contains a chimeric Tn5 Km^r gene sequence, from the plasmid vector pCIB10 (Rothstein et al., 1987) was obtained from J.M. Davis. The second was cosmid DNA, isolated from pFW302 (covering T_L-DNA) by the alkaline lysis method described by Ausubel *et al.*(1987). Cosmid DNA was digested with the restriction enzyme HindIII as specified by the manufacturer (Boehinger Mannheim). Probe DNA was radioactively labeled using a random primed labelling kit obtained from DuPont with [^{32}P]dCTP (3000 Ci/mmol (1 Ci = 37 GBq); New England Nuclear). Hybridization and washing were carried out as specified by Bio-Rad for Zeta-probe membrane. The blots were visualized by exposure to Kodak XAR-5 film with an intensifying screen at -70^{o} C for 2 to 10 days.

RESULTS

Plant transformation. Hypocotyl segments inoculated with *Agrobacterium rhizogenes* strains R1500 and R1601 (Pythoud *et al.*, 1987) developed adventitious roots after 4 days culture on MSO medium (Figure 1). No significant effect of seedling age was found for adventitious root formation. The hypocotyl segments inoculated with *A. rhizogenes* R1601 showed a higher percentage (18%) of hairy root formation than those inoculated with R1500 (10%) (Table 1). The addition of kanamycin (100ug/ml) in MSO after the 3 day cocultivation period significantly lowered the percentage of explants exhibiting tumorous roots ($P < 0.01$, Table 2). Significant differences in the number of hypocotyl segments producing hairy roots on MSO without kanamycin were observed between explants treated with and without IAA (Table 2). However, the addition of 2,4-D did not affect transformation frequency (Table 2). The highest percentage (42%) of transformation was achieved from explants which were inoculated with *A. rhizogenes* R1601 and cultured on MS medium in the presence of 1 uM IAA (Table 2).

Shoot regeneration and selective culture. After transfer to callus induction medium (BCM), containing 100 ug/ml kanamycin, Ri-induced hairy roots exhibited distinctive traits. They were fast growing, larger, and showed a

Table 1. Effect of seedling age on percentage of explant which produced adventitious roots after 4 weeks of culture following inoculation with *Agrobacterium rizogenes* strains R1500 and R1601.

Seedling age (days)	Strains	# explants	% root
5	R1601	73	24.7
	R1500	70	10.0
	Control	85	0.0
6	R1601	22	18.2
	Control	25	4.0
10	R1601	28	17.9
	Control	70	1.4
11	R1601	43	11.6
	Control	21	4.8
25	R1601	30	10.0
	Control	24	0.0
Total	R1601	196	17.9
	R1500	70	10.0
	Control	225	1.3

Table 2. Effect of exogenous auxin and kanamycin in MS medium following co-cultivation with *A. rhizogenes* on the formation of hairy root.

Medium1)	Strain	# explants	% root2)
MSO	R1601	196	17.9
	R1500	70	10.0
	Control	225	1.3
MSO + 1 uM IAA	R1601	76	42.1
	Control	71	38.0
MSO + 1 uM 2,4-D	R1601	42	9.5
	Control	20	5.0
MSO + 100 ug/ml Km	R1601	110	5.5
	Control	73	2.9
MSO + 1 uM IAA + 100 ug/ml Km	R1601	114	4.4
	Control	50	2.0

marked development of lateral roots (Figure 1).

Roots began to form callus in less than a month. While the calli remained white for about two months on callus culture medium (BCM), with kanamycin, they eventually turned green (Figure 2).

In the process of forming callus, a small proportion (less than 5%) of adventitious roots gave rise to spontaneous shoots (Figures 3 and 4). Shoots regenerated from root tumors showed the same growth and proliferation pattern on the shoot culture medium (BSM) containing 100 ug/ml kanamycin as normal shoots derived from *in vitro* bud culture did on BSM without kanamycin (Figure 5). However, the transformed shoots produced highly proliferating roots on BSM, while normal shoots do not root on BSM (Figure 6). In contrast to the healthy growth of transformed shoots on selection medium, the leaves of normal shoots in the presence of 100 ug/ml kanamycin turned yellow and eventually abscised (Figure 5).

The results of the rooting test for putative transformed shoots in black locust rooting (BRM) medium (Han and Keathley, 1991) are shown in Table 3. Although all 10 putative independent transformants remained green in the presence of 100 ug/ml kanamycin, only 5 rooted. Based on the rooting performance of transformants #771, 772, and 773, no significant differences in rooting percentage were observed for these lines between shoot culture medium (BSM) and rooting medium (BRM) except for #771 (Table 3). Control shoots did not produce roots or survive on the selection medium.

Further testing for the expression of kanamycin resistance in the transformed plants was conducted by culturing leaf pieces on callus induction medium (BCM) containing 100 ug/ml kanamycin. All of the leaf pieces from the putative transgenic shoots produced callus on the selective BCM (Table 4; Figure 7). The leaf pieces also showed shoot and root morphogenesis on the same medium and each independent transformant showed an individual response (Table 4). Leaf pieces from control plants did not survive the kanamycin medium but did produce callus on MSO (Figure 7).

Acclimation of transgenic plants to *in vivo* condition: Transformed black locust plantlets were potted (Figure 12) and maintained as described in Han *et al*. (1990). Within two weeks after potting, 20 to 30% of the transformed plants died. This represents a higher than normal mortality rate when compared to normal, non-transformed plants. However, until a certain stage of development, about 3 months after potting, the remaining plants were more vigorous, fast growing, and had leaves that were thicker and darker than normal shoots. After 3 months of growth, when the average shoot height reached 60-70 cm, phenotypic alterations in

Table 3. Rooting of transformed black locust shoots after 4 weeks of culture in the presence of 100 ug/ml kanamycin.

Lines	Medium	# shoots	% root	Color
771	BSM +Km	14	100.0	Green
	BRM +Km	7	42.9	Green
772	BSM +Km	28	7.1	Green
	BRM +Km	10	20.0	Green
773	BSM +Km	66	63.6	Green
	BRM +Km	12	66.7	Green
774	BSM +Km	5	0.0	Green
776	BSM +Km	2	100.0	Green
777	BRM +Km	2	50.0	Green
891	BSM +Km	11	0.0	Pale-Green
	BRM +Km	8	0.0	Green
941	BSM +Km	3	0.0	Green
942	BSM +Km	2	0.0	Green
943	BSM +Km	8	0.0	Green
	BRM +Km	3	0.0	Green
944	BSM +Km	1	0.0	Green
Control	BRM +Km	25	0.0	White / Abscised

Table 4. Callus induction from leaf pieces of transformed black locust shoot after 4 weeks of culture in MS + 100 ug/ml kanamycin.

Lines	Explants No	Callus %	Root %	Shoot %	Color[1)]
771	44	100.0	59.1	0.0	G
772	34	100.0	8.8	5.9	G,GW
773	37	100.0	5.4	2.7	G,GW
774	10	100.0	10.0	0.0	G,GW
920DS	49	49.0	0.0	0.0	G
Control	20	0.0	0.0	0.0	W,Y,D
Control -Km	20	100.0	0.0	0.0	G

1) Color: G-green, GW-greenish white, W-white, Y-yellow, D-dark brown (died).

leaf morphology were observed (Figures 8 - 11). The abnormalities in leaf morphology included: wrinkled, furrowed, and variegated leaves, as well as asymmetrical leaflets. Each transformant seemed to have a different aberrant phenotype in leaf morphology. For instance, wrinkled leaves were found in all transformants, variegated leaves were in #772, and furrowed leaves in #773. The root system of potted transformants was larger than that of non-transformed plants (Figure 13).

Southern analysis. DNA was readily isolated from each of three independent transformants, #771, 772, and 773, using the protocol described in above. After digestion with restriction enzyme HindIII, the DNA was size fractioned by electrophoresis and transferred to a Zeta-probe membrane following the manufacturer's (Bio-Rad) instructions. The filter bound DNA was then hybridized to ^{32}P-labeled probes (either pFW302 which represent T_L-DNA or the PstI fragment which contains the Km^r gene). All three transformants contained fragments which comigrated with fragments 17, 30, and 32 from HindIII digestion of pFW302 (Figure 14a). These results suggest that the T_L-DNA sequences were present in the transformants. The fragment comigrating with HindIII fragment 0.8 was not found in this hybridization pattern. The absence of a fragment comigrating with HindIII fragment 21 was not surprising because

R1601 has a transposon insertion in this fragment (Sinkar et al., 1987). Although no fragments that comigrated with T_L-DNA fragments were found, some hybridizing bands were present in the control DNA lane. The reason for this hybridization is not currently clear. But, this might suggest that there are homologous sequences between black locust and cosmid (pFW302) DNA.

Hybridization patterns using the *NPT* II gene as a probe are shown in Figure 14b. The DNA of all transformants showed bands which hybridized to the probe. No detectable bands were found in control lane.

DISCUSSION

Tree species are generally more recalcitrant to genetic transformation and subsequent regeneration of transgenic plants than herbacious species. The successful transformation and regeneration of transgenic plants requires well defined *in vitro* culture conditions for the particular species. Several woody species transformations have been reported in both gymnosperms (Dandekar et al., 1987; Karnosky et al., 1988; Loopstra et al., 1990; Sederoff et al., 1986) and angiosperms (Baribault et al., 1989; Davis and Keathley, 1989; Guellec et al., 1990; Kobayashi and Uchimiya, 1989; Mackay et al., 1988; Vahala et al., 1989). But until now the regeneration of transformed plant has been reported in only 4 woody species, including poplar (Fillatti et al., 1987; McCown et al., 1991; Pythoud et al., 1987), walnut (McGranahan et al., 1988), apple (James et al., 1989), and grape (Mullins et al., 1990).

The results presented in this chapter clearly demonstrate genetic transformation and subsequent regeneration of transformed black locust. Thus, black locust becomes the fifth woody plant species to be regenerated from transformed tissues.

Black locust shoots have been regenerated from hairy root tissues in the presence of kanamycin and subsequently cultured on selective medium using kanamycin. Rooting of regenerated shoots on selective medium was a quick assay for the expression of the kanamycin resistance gene in the plant. All of the shoots from transformant #771, 772, and 773 produced roots on BSM supplemented with kanamycin. To further confirm the expression of kanamycin resistance, leaf pieces from individual transformants were cultured on callus induction medium containing kanamycin. All the leaf pieces induced callus while normal leaf pieces died. Abnormal phenotypes in leaf morphology observed among the transformants also confirm transformation because these abnormalalites are closely correlated to the expression of T_L-DNA *rol* genes (Cardarelli *et al.*, 1987; White *et al.*, 1985). And finally, the molecular evidence for transformed black locust comes from Southern analysis, which demonstrated the presence of sequences from both the T_L-DNA region and the kanamycin resistance gene in the transformants.

This research was successful for three main reasons; 1) The basic knowledge of tissue culture procedures, including the dedifferentiation and regeneration of black locust, had been well established (chapters 1 and 2). 2) Use of the proper vector system. We also tried a disarmed Ti-plasmid (pGV3850HPT::pKU2, Baker et al., 1987) which was not successful in getting transgenic black locust. This is not too surprising since vector specific effects are also found among Ti-plasmids (Guri and Sink, 1987). 3) Use of proper plant materials in terms of species, genotype, and juvenility. We have found that black locust can be easily manipulated *in vitro* (Han and Keathley, 1988, 1989; Han et al., 1990; see also previous chapters). We also used explants from juvenile materials.

When IAA was added to the medium on which explants co-cultivated with *A. rhizogenes* were incubated, the percentage of explants that produced hairy roots was significantly increased. This result is interesting in relation to the recent findings that have determined that hairy root formation is not the result of the activity of the T_R-DNA borne auxin gene or due to a substantial imbalance of endogenous phytohormones, but it is a result of an increased sensitivity to auxin conferred by the T_L-DNA (Cardarelli et al., 1987; Shen et al., 1988; Spano et al., 1988).

Since a high concentration of kanamycin was used (100 ug/ml; most other reports used 5-50 ug/ml), *in vitro* selection was very efficient, all normal plants were killed and transformants were still able to undergo growth and organogenesis. These results suggest that there is enough NPTII activity in the transformed tissues to allow growth and organogenesis. The level of kanamycin being used is important because if it is too low, the selective culture might end up with untransformed escapes. Consequently, if it is too high, then the selective culture might kill transformants which have a low level of NPTII enzyme activity. The concentration of kanamycin used in the present studies is suggested for future transformation studies involving black locust.

Typical phenotypes of hairy root derived plants have been described as having dark wrinkled leaves, an extremely abundant and plagiotropic root system, reduced apical dominance, reduced internode length and leaf size, and the ability of leaf explants to differentiate roots in a phytohormone-free medium (Cardarelli et al., 1987; Hamamoto et al., 1990; Spano et al., 1987; Tayler et al., 1985; Tepfer, 1983, 1984). Reduced apical dominance, internode length, and leaf size were not found in black locust transformants. In all three transformants the hairy root phenotype was not expressed at the early shoot growth stage, but rather became evident after 3 months growth.

Interestingly, in addition to the wrinkled leaf morphology, asymmetrical and variegated leaves were found in black locust transformants. These phenotypes were not reported earlier as a hairy root phenotype, but Kim et al. (1973) found the same abnormalities in their black locust breeding efforts which utilized mutations generated by X-ray and thermal neutrons. Therefore, these abnormalities have possibly originated from the genomic disturbance due to the insertion of foreign DNA, rather than from the expression of T-DNA genes in the transformants. This hypothesis might explain why each transformant has a different abnormal phenotype in addition to wrinkled leaves which is closely correlated to the presence of the T_L-DNA *rol*B locus (Cardarelli *et al.*, 1987; White *et al.*, 1985). Southern analysis, utilizing pFW302 (covering T_L-DNA) as the probe, showed the presence of T_L-DNA in all three transformants. The absence of a fragment comigrating with the HindIII 0.8 fragment of pFW302 in the hybridization pattern may suggest incomplete incorporation of T-DNA. But it remains to be studied further.

In summary, the transformation and regeneration system reported here is repeatable and effective, allowing routine introduction of genes into the black locust genome. Successful use of this system may provide the development of locust borer resistant and/or spineless black locust, which substantially saving time and effort, when compared to typical tree improvement program.

Figure 1. Culture of hairy roots derived from hypocotyl segments which cocultivated with *Agro bacterium rhizogenes* R1601.

Figure 2. Cultures of hairy root-derived callus on BCM supplemented with 100 ug/ml kanamycin.

Figure 3. Spontaneous shoot regeneration from hairy root cultures on BCM containing 100 ug/ml kanamycin.

Figure 4. Spontaneous shoot regeneration from hairy root cultures on BCM containing 100 ug/ml kanamycin.

Figure 5. Shoot cultures regenerated from hairy root tissues of black locust.

Figure 6. Rooting of shoots regenerated from hairy roots on BSM containing 100 ug/ml kanamycin.

Figure 7. Cultures of leaf pieces on callus induction medium in the presence of 100 ug/ml kanamycin.

Figure 8. Abnormal phenotype in leaf morphology in transformant #771.

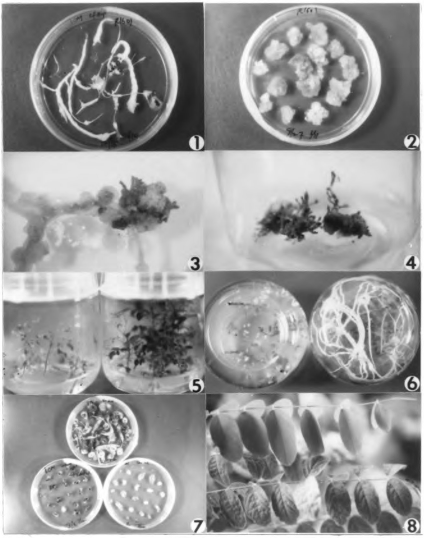
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Figure 9. Abnormal phenotypes in leaf morphology in transformant #772.

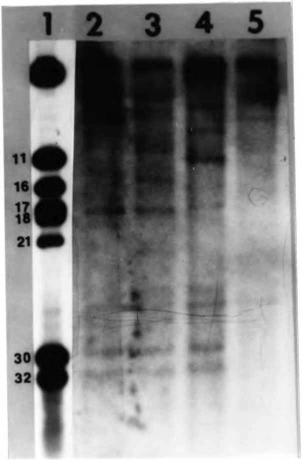
Figure 10. Abnormal phenotypes in leaf morphology in transformant #773.

Figure 11. Potted transformed black locust (#771).

Figure 12. Potted transformed black locust (#773).

Figure 13. Root systems of both normal and transformed (#771) black locust.


Transgenic
Normal
9
10
11
12
13



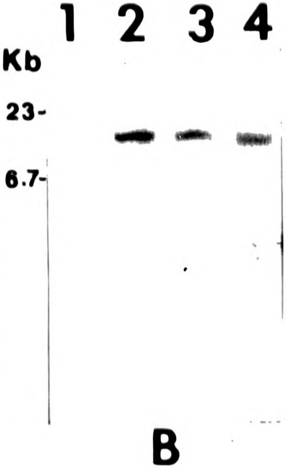


Figure 14a: Southern blot analysis of transformed black locust plants. Total black locust DNA was digested with HindIII, size fractioned on agarose gel by electrophoresis and transferred to Zeta-Probe membrane (Bio-Rad). Southern blots were hybridized with ^{32}P-labeled pFW302 DNA (covering T_L-DNA). Lanes: 1) DNA (10 ng) from pFW302; 2) transformant #771 (0.7 ug); 3) transformant #772 (2 ug); 4) transformant #773 (6 ug); 5) DNA (3 ug) from untransformed black locust. Molecular size markers are fragment numbers from HindIII digestion of pFW302 (Taylor *et al.*, 1985).

Figure 14b: Southern blot showing hybridization of probe (1 kb PstI fragment from pCIB10 (Rothstein *et al.*, 1987) to DNA from transformed black locust. Plant DNA was digested with HindIII. Lanes: 1) DNA from untransformed black locust; 2) from transformant #771; 3) from #772; 4) from #773.

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CONCLUSION AND RECOMMENDATIONS

Application of plant genetic engineering technology in a black locust improvement program holds great promise for accomplishing specific breeding goals such as insect resistance or spinelessness. The results of the experiments described in this dissertation clearly show the feasibility of the application of such technology into ongoing tree improvement programs.

The results of the experiments described in the first chapter illustrate that the regeneration of black locust can be achieved from callus tissues derived from either hypocotyl or internodal segments of *in vitro* shoot cultures. This plant regeneration system was simple and repeatable. Whole plant regeneration from unorganized tissues is therefore no longer a barrier for genetic manipulation of black locust at the cell or molecular level.

This system can be utilized in many ways in tree breeding efforts such as *in vitro* selection, somaclonal variation, somatic hybridization, somatic embryogenesis, and genetic transformation. Since the successful application of these techniques relies on the regenerability of manipulated cells and tissues, practical application of this system should be considered at the early stages of a breeding program if biotechnology is to be employed.

The results of the experiments described in Chapter 2 indicate that cambial tissue of mature black locust can be used to generate callus and from this callus shoot regeneration is possible. It was important to be able to regenerate shoots from mature trees, because *in vitro* techniques have been confined to seedling materials (Bonga, 1982) and this limitation has been an unsolved problem in the application of biotechnology as an alternative to traditional methods. Now, it is clear that direct use of mature trees as starting materials in *in vitro* culture, and manipulation of tree species is feasible by using cambial tissues. Furthermore, cambial tissues are available regardless of season so that the culture can be initiated year-around.

The results described in Chapter 2 also demonstrate that the cambial tissues can be stored without substantial lose of regenerability. The ability to store cambial tissues has two practical applications: 1) in the short term, it facilitates testing and the development of tissue culture systems by simplifying the logistics of the process, and 2) cambial tissues could be preserved in tissue banks for conservation of tree germplasm by either cryopreservation or cold-room storage methods.

The results described in Chapter 3 report the regeneration of transformed black locust. It is important to report this, because there are currently only 4 woody species (apple, grape, poplar, and walnut) capable of regenerating transformed plants.

Hypocotyl segments inoculated with *Agrobacterium rhizogenes* produced hairy roots on phytohormone-free medium. Transformed plants were obtained by spontaneous shoot regeneration from hairy root tissues cultured on callus induction medium containing kanamycin. Consequently, shoot regeneration from transformed callus has not been attempted. Regenerability of transformed callus remains to be tested. All transformants clearly showed *rol*B phenotype such as wrinkled leaf, remarkably abundant root development, and rooted on phytohormone-free medium. It is of interest to note that only the explant inoculated with Ri-plasmid vectors produced transgenic trees. Therefore, the use of an Ri-plasmid based vector system may be recommended for future transformation work with black locust.

With this Ri-plasmid mediated gene transfer system, genetic manipulation of certain traits, such as locust borer resistance or spinelessness, is possible. However, since this system utilizes seedling materials, it currently has limited use in a breeding program. The development of a protocol for the transformation of mature cambial tissues utilizing this transformation system will provide the means to overcome the limitation of using seedling materials.

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