Optimizing Energy Yields in
Black Locust
Through
Genetic Selection

Final Report

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OPTIMIZING ENERGY YIELDS IN BLACK LOCUST THROUGH GENETIC SELECTION

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ABSTRACT

In 1986, black locust (*Robinia pseudoacacia*) was selected as one of five "model" taxa for development as a woody biofuels crop. Although black locust had shown promise in a number of locations, its potential on agriculturally marginal sites, which were expected to supply a large and ready land base for biofuels plantations, was emphasized. In the Piedmont of the southern states, especially, there was an abundance of agriculturally degraded land on which the juvenile growth rate of black locust had surpassed that of other candidate species in early trials. This project was initiated to determine whether productivity of black locust on such sites was sufficient to sustain a viable crop. The answer to that question was to be based on yields generated following genetic improvement.

To predict the outcomes from genetic improvement, several kinds of information were sought. These included: 1) the amount and geographic pattern of natural genetic variation, 2) the mating system of the species, 3) quantitative genetic parameters of relevant traits, 4) the relationship between nitrogen fixation and growth in black locust, 5) the viability of mass vegetative propagation, and 6) the feasibility of improvement through genetic transformation.

Black locust was found to possess copious natural genetic variation in biomass production under short-rotation, intensive-culture conditions. We estimate that biomass could be increased by more than 50% over unselected stock in a single cycle of selection and testing. Gains of 100% or more are theoretically possible with vegetative propagation, however, mass vegetative propagation remains difficult in this species. Biomass yields can also be increased by 10% or more with the application of nitrogen fertilizer, even though black locust acquires most of its nitrogen from symbiotic fixation. Fertilization at the beginning of the growing season is expected to provide the greatest benefit. Depending on site conditions, biomass yield following the first generation of genetic selection is predicted to range from 5 to 10 Mg ha⁻¹ yr⁻¹ on eroded Piedmont uplands. This is well short of the desired 20 Mg ha⁻¹ yr⁻¹. Part of the downfall stems from early leaf abscission, apparently caused by leaf pathogens, including black locust anthracnose, *Colletotrichum gloeosporioides*. Nevertheless, heavy soil texture is probably the greatest impediment to high productivity. Growth of black locust was very rapid during the first two field seasons, but then began to slow. Similar observations have been made elsewhere when black locust was grown on clayey soils. Root excavation studies suggest that root growth is inhibited in clay soils, thus constraining shoot growth.

Genetic transformation offers a promising avenue to alleviate pathogen and insect problems that reduce biomass production. Work thus far has shown that somatic embryos can be produced, and in them transient gene expression has been achieved using microprojectile bombardment. Because of the significant impact of insect and disease pests on black locust productivity, continued development of genetic transformation methodologies should be a high priority. Furthermore, the growth of black locust on sites with lighter textured soils should be examined to determine the true merit of this species as a biofuels crop. Judgements based on performance on clayey, degraded soils will be unfairly negative.

OPTIMIZING ENERGY YIELDS IN BLACK LOCUST THROUGH GENETIC SELECTION

INTRODUCTION

Objectives

The purpose of this work was to assess the magnitude of improvement in biomass yield of black locust possible through breeding, and to determine methods for efficiently capturing the yield improvement achievable from selective breeding.

To meet this overall objective, six tasks were undertaken:

1. Determine geographic patterns of genetic variation that exist among native black locust populations. This information is needed to establish initial breeding populations with the most desirable native material.
2. Determine the mating system of black locust. Knowledge of mating system is required for choosing effective breeding strategies and for accurately estimating genetic parameters used to select superior trees and estimate improvement.
3. Determine the magnitude of genetic parameters including variances, heritabilities and correlations among traits which are components of energy yield. These parameters are needed to construct efficient breeding plans.
4. Determine the relationship between growth rate and amount of nitrogen fixation. This information is needed to determine whether breeding should focus on black locust grown under high nitrogen or low nitrogen conditions.
5. Develop methodologies for mass vegetative propagation of black locust. In most cases, vegetative propagation will provide opportunity for greater genetic improvement than will propagation by seed.
6. Develop methods for genetic transformation in black locust. Such methods may be required for introduction of traits which may not exist within the native gene pool, including resistance to insects and diseases.

In addition to these tasks, information on the culture of black locust under short-rotation, intensive management has been obtained. Further, selections of superior black locust for biomass production have been identified.

Background

In 1986, following a decade of extensive species testing, five promising "model" woody taxa were chosen by the USDOE Biofuels Feedstock Program for closer examination and development as short-rotation woody biomass fuel crops. One of these was black locust, *Robinia pseudoacacia*. Black locust had shown very rapid juvenile growth in a variety of geographical settings, including Michigan, Kansas and Georgia. Furthermore, its high specific gravity (the highest of the species considered) is advantageous in reducing transportation and storage costs. Its ability to excel in

adverse soil conditions, largely through fixation of atmospheric nitrogen, however, was the attribute that clinched its selection as one of the five model taxa.

Black locust has long been identified as the species of choice for revegetating land reclamation sites, such as mine spoils. Such sites are typically deficient in soil nitrogen, and often have excessively high or low pH's and/or some mineral anomaly. Under these conditions, black locust has consistently survived better and grown faster than other tree species. Because marginal and degraded lands represent a large land base potentially available for biofuel plantations, interest in black locust was focused on its capacity for production on such sites.

One region of the country which has an abundance of degraded, marginal lands is the Piedmont of the southeastern States. Once prime for cotton and corn, this province has undergone serious erosion, exposing the clay subsoil and rendering much of the region unprofitable for traditional agriculture. Previous work already suggested that black locust's juvenile rate of growth on eroded Piedmont sites exceeded that of other native (*Liquidambar styraciflua*, *Platanus occidentalis*, *Liriodendron tulipifera*) and exotic (*Alnus glutinosa*) fast-growing species. However, it was not clear what level of production was attainable. This work was initiated primarily to answer that question.

Biomass yield is, of course, not a single value, but a function of cultural conditions and genetic stock. The central purpose in this project was to determine yields possible following genetic selection, using the "best" cultural practices currently known. Genetic improvement is stressed because it represents an untapped source of increased productivity which, based on experience with other agricultural crops and forest trees, is potentially substantial. This, in turn, requires that a breeding strategy be formulated. Our aim, therefore, was to examine the feasibility and efficacy of alternative breeding strategies, and to estimate yields possible from the "optimal" one.

Format of Report

This report reviews the products of research conducted during the seven-year life of this project. Much of the work has been reported in published technical journals, the most important of which are reproduced in the appendix. Only highlights are given here, although greater detail is provided for results not yet described elsewhere.

SPATIAL PATTERNS OF GENETIC VARIATION

Geographic patterns of genetic variation were studied on two levels --1) range-wide (native range) and 2) within stand (10 ha watersheds).

Range-wide variation was studied using allozymes and nursery height and diameter measures. The purpose of these studies was to determine if genetically related black locust populations are geographically clustered. Clustering may occur because of geographic patterns in natural selection or because of common ancestry in neighboring populations. In most tree species, nursery growth is correlated with growing season length and is considered adaptive, whereas allozymes act normally like neutral markers indicating common ancestry. Often, though, it is not possible to differentiate between the alternative causes of clustering. Nevertheless, knowledge of geographic variation patterns is valuable from a practical perspective. For example, evidence of geographic genetic variation in growth traits argues for establishment of an initial breeding population composed largely of selections from the observed optimal region(s). If populations cluster only with respect to allozyme frequencies, efforts should be made to sample from each cluster, as each may represent a different genetic heritage that should be included to maximize diversity and the potential for genetic improvement.

Variation within 10 ha watersheds was studied with allozymes to determine genetic structure among individuals within stands. Specifically, we sought to determine the relative abundance of sexual and asexual reproduction, and the spatial extent of clones produced via root suckers. This information is needed to minimize the potential for selecting multiple stems of the same clone during establishment of the initial breeding populations.

Range-Wide Variation

Nursery Growth

Open pollinated seeds from 145 black locusts from throughout the native range were collected over three years. Only one tree per stand was sampled to avoid multiple collections from the same clone. However, sample stands were clustered in groups of two to eight occurring within a circle of 10 mile radius. Each group is referred to as a "population" for the remainder of this report.

Seedlings were grown using the following protocol, designed to minimize environmental variation. Multiple seeds were sown in 40 cm³ containers/tubes in a greenhouse. Each family was represented by 98 containers, placed in a single rack, in each of two blocks. Shortly after germination, seedlings were culled to one per container. Containers were then evenly spaced among two racks per family per block, each holding up to 49 containers. Within a rack, seedlings were arranged by size so that larger early germinants would not shade smaller late germinants. Seedlings were grown for six weeks in the greenhouse, during which racks were rotated on a daily basis in an effort to minimize within greenhouse environmental effects. They were then moved to a lathehouse for acclimation to outdoor conditions, and finally transplanted to an outdoor nursery at 15 x 15 cm spacing. Each family was represented by one plot of 18 trees (3 rows of 6 trees) in each of four nursery blocks.

Height and root collar diameter were measured on each tree. Environmental variation within a nursery block was modeled using spline regression (Bongarten and Dowd 1987), and the deviations used as observations for analysis.

Families ranged from 152 to 56 cm in mean height and from 12 to 5 mm in mean diameter. Twenty to 25 % of the family variation was among populations, the remaining 75-80% accounted for by family variation within populations (Table 1)

TABLE 1. Variance of height and diameter in black locust nursery seedlings, and the percentage of the variance attributable to various sources.

Source of Variation	Height	Diameter
	(%)	(%)
Population	2.9	1.9
Family (Population)	8.9	7.2
Plot (Family)	13.5	7.3
Within Plot or Error	74.6	83.6
Total	2180.4 cm ²	11.73 mm ²

Unlike several previous range-wide nursery studies with black locust (Kennedy 1983, Mebrahtu and Hanover 1989, Genys and Harman 1990), including one of our own, mean family growth measures were significantly correlated with latitude, longitude and elevation of origin. Height and diameter decreased as origin was increasingly northern, with latitude explaining 5-7% of the variation in family means. Height and diameter were also generally greater in trees from western and low elevation populations. However, this may occur solely because western and low elevation populations were largely confined to southern latitudes. Multiple regression showed no influence of longitude or elevation on seedling size once the effect of latitude was taken into account. Geographic patterns of variation are shown using population means for height in Figure 1.

FIGURE 1. Mean height of black locust seedlings from native populations as a function of latitude and longitude of origin.

Our detection of significant correlation between latitude and growth rate stands apart from other similar studies, but it is probably a realistic representation of geographic variation patterns, facilitated by stringent efforts to control environmental variation.

Allozymes

Allozymes were examined in a portion of the same families grown in the above-mentioned nursery study. A complete description of that work was published in Surles et al 1989, and is reprinted in the Appendix.

Major findings of the allozyme analysis were:

1. Allozyme variation in black locust is among the greatest found in any tree species.
2. The overwhelming majority of allozyme variation (95%) resides within populations.
3. There is no evidence of geographic clustering among like families or populations.
4. Populations from the margin of the species' natural range had the most divergent allozyme frequencies.

Conclusions

Both the latitudinal trend in growth rate and the divergence of marginal populations in allozyme frequencies are typical of native populations of tree species. These results provide evidence that, despite massive planting with non-local stock (Bongarten 1992), at least vestiges of the natural geographic patterns of genetic variation patterns remain within the native range.

In selecting trees for a base breeding population, southern populations should be given the greatest emphasis because of the tendency for southern trees to grow more rapidly than northern trees. However, latitude accounts for only about 6% of the variation in family growth rate, so there is ample justification to consider selections from throughout the range, especially if they are found to have other valuable characteristics (such as straight stems).

Within Stand Genetic Structure

Within stand genetic structure was examined in two 10 ha watersheds in the Coweeta Basin in western North Carolina. Both stands have been without human disturbance for the past 70 years. Previously, logging had been practiced, but the extent of that logging is not known. One watershed (no. 2) faced south, and the other (no. 21) faced north. Both were at about 1000 m elevation and were separated from each other by about 4 km. The south-facing watershed was drier and had less canopy coverage than the north-facing watershed.

In both stands, an attempt was made to identify all black locust stems. To aid in the search, a 10 x 15 m string grid was established on each watershed. Each of the grid cells was then surveyed for black locust stems. In Watershed 2, 749 stems were found, of which 640 were "sprouts", less than 1 cm in diameter. In Watershed 21, 245 stems were found of which 161 were in the sprout class.

The allozyme genotype of each stem over 1 cm diameter and half of the sprouts was determined using 8 polymorphic loci. Stems with common allozyme genotypes were assumed to belong to the same clone. The probability that stems having the same allozyme genotypes were members of different clones was generally less than 1%.

Asexual vs. Sexual Reproduction:

On Watershed 2, 16 clones were identified, and these accounted for 93% of the stems over 1 cm and 90% of the sprouts. On Watershed 21, 15 clones were found; they accounted for 100% of the large stems and 96% of the sprouts.

Spatial Distribution of Clones:

Members of a clone generally clustered tightly together, and clones rarely interdigitated. However, three clones in each watershed were composed of two or three disjoint clusters of stems (e.g. Figure 2). On average, the distance between the most widely separated members of the same clone was 75 m, but the range among clones was 20 to 350 m. Clones with the widest dispersion among members were those composed of multiple disjoint clusters. No single clonal cluster was more than 100 m in its greatest dimension.

FIGURE 2. Spatial distribution of stems of clones found on Watershed 2.

Relatedness Among Clones:

Genetic similarity among clones was measured by number of alleles in common. The maximum number of alleles in common was 16, two for each of eight loci. The average number of alleles in common among clones in Watershed 2 was 12.8. For Watershed 21 it was 10.0. And between clones in the two watersheds it was 10.8. Thus, on Watershed 2, clones showed significant familial relatedness, whereas clones on Watershed 21 appear to be unrelated.

Demography and Genetic Structure:

These results show that black locust, in stands typical of the Appalachians today, exists largely in clonal patches less than 100 m in width. New stems are produced primarily from root suckers, but reproduction by seed is also evident. Most new stems do not survive long enough to reach 1 cm in diameter. Only where light is abundant will new stems grow and persist. Examination of stem diameters (Figure 3) shows that new black locust stems are being recruited on a continuous basis in the sunnier south-facing watershed, whereas new recruitment in the north-facing watershed has been negligible since the post-logging cohort was established 70 years ago.

FIGURE 3. Frequency distribution of black locust stem diameters in a moist, north-facing watershed (21) and a drier, south-facing watershed (2).

Based on size distribution of stems and relatedness among clones, we suggest that black locust is a more-or-less permanent fixture on dry south-facing slopes and ridge tops, where fire and sparse canopy provide regular opportunities for the establishment of new stems derived from root suckers or seeds of the trees existing on site. Recruitment of black locust on moist, north-facing slopes occurs only after a major disturbance. In the intervals between disturbances black locust is probably eliminated from the stand, and new unrelated cohorts are established at the time of each disturbance.

The clonal nature of black locust and relatedness among nearby clones, especially on drier sites, must be considered when selecting trees from natural stands for a breeding program.

MATING SYSTEMS

The mating system of black locust was examined using open-pollinated seeds of trees from natural stands. A sample of the seedlots used to determine range-wide variation patterns (described above) were employed.

The degree of outcrossing/inbreeding and the genetic correlation among family members was inferred from allozyme data. Results of this work were reported in two papers (Surles et al 1990a and Surles et al 1990b), which are reprinted in the Appendix.

The most important findings of these investigations are as follows:

1. In nature, black locust is primarily, but not exclusively, an outcrossing species. Average outcrossing rate was 0.83.
2. Outcrossing rate varies greatly among families, ranging from less than 0.5 to 1.0.
3. Mechanisms that enforce outcrossing are not fully known, but protogyny appears to be an important factor.
4. Family germination rate was negatively correlated with inbreeding rate, suggesting that inbreeding has debilitating effects in black locust.
5. Most open-pollinated families fall between half-sibs, which have a quarter of their genes in common, and full-sibs, which have half of their genes in common. On average, members of open-pollinated families have about a third of their genes in common.
6. As with degree of outcrossing, black locust families varied widely in relatedness among family members from true half-sibs to nearly true full-sibs.

These results show that black locust should be treated as an outcrossing species when designing breeding strategies. Inbreds will be difficult to produce and show depressed growth and morphological anomalies.

Furthermore, because open-pollinated families differ greatly in their genetic composition, selection of superior individuals from open-pollinated progeny tests, which are favored because of the difficulty in producing seeds from controlled crosses, will be complicated.

PROGENY TESTING

An open-pollinated progeny test was established to provide estimates of genetic parameters that would be used to design optimal breeding strategies and to estimate progress that could be made through selective breeding. Superior individuals in the test could also be identified and incorporated in improved breeding populations.

The primary parameters to be estimated were 1) additive genetic variance and heritabilities of yield and its component traits, 2) genetic correlations between yield and its component traits, 3) juvenile-mature correlations, and 4) genotype x environment interaction effects.

Inclusion of irrigation and nitrogen treatments also provided valuable information on the culturing of black locust. Furthermore, observations made in the progeny test revealed that the growth of black locust is very sensitive to soil conditions, and that leaf pathogens may significantly reduce yields.

Design Considerations:

The progeny test was established on a single site to control costs. However, environmental variation was induced with irrigation and nitrogen fertilization treatments, and by planting in two years. Furthermore, the test site, itself, was heterogeneous, as evidenced by large differences in growth rate among the blocks. We view the results as generally valid for Southern Piedmont uplands. Companion tests have been established in West Virginia and Kansas, but data from those locations are not reported here.

Family plot size in the progeny test was 24 trees (6 x 4). This was a compromise between smaller plot designs in which family variance is inflated by competition between families, and larger plot designs which offer poor statistical precision. Gains in yield per hectare from family selection could be reasonably estimated, but the additional improvement from individual tree selection have lower reliability.

Results:

Results from the progeny test were reported in Bongarten et al 1992 and Bloese et al 1992, which are reprinted in the Appendix. The most important findings were:

1. Irrigation and nitrogen fertilization both increased growth on eroded Piedmont uplands which are droughty and nitrogen poor. Irrigation increased growth by 50% and nitrogen fertilization increased growth by 10%. Their effects were additive.
2. Family differences in yield were very large. The best families had 300% more yield than the worst. From family selection alone (best 1 in 20), yield can be increased by 50% over the mean. With individual selection, it is not unreasonable that yield could be doubled.
3. No genotype x cultural treatment effects were evident. The best families were best under all cultural conditions.
4. Genetic variation in juvenile specific gravity is negligible. Virtually all of the variation in yield stems from variation in volume alone.
5. Growth rate of black locust is extremely rapid in the first two years, comparing favorably with hybrid poplars grown on much better sites. However, on eroded Piedmont uplands, growth begins to slow by the third or fourth year. Rotation age should not be more than five years.
6. Growth of black locust appears to be very sensitive to soil aeration and rootable depth. Trees died in depressions where water was allowed to stand, and growth was substantially greater where soils were loose and drainage better.

7. By using seedlings from the best families, dry matter yields on upland Piedmont sites are expected to be between 3 and 8 mg ha⁻¹ yr⁻¹, depending on site conditions. Yields will be greater when seeds from matings of selected trees are used, perhaps by as much as 50%. It is difficult, however, to estimate yield improvement per acre from individual tree measures. In any event, it is unlikely that the 20 mg ha⁻¹ yr⁻¹ goal can be reached on eroded Piedmont uplands, unless novel treatments can be found to improve growth.

Two novel treatments which may improve growth are 1) the addition of calcium sulfate and 2) the introduction of pathogen resistance through genetic transformation.

Amelioration of Soil Conditions:

Additions of calcium sulfate at rates between 4 and 10 tons per acre have been shown to increase the yield of agricultural crops, such as alfalfa, on clayey Piedmont uplands by 300% (Sumner et al 1989). The mechanisms behind the yield improvement are not fully understood, but soil structure appears to be improved, leading to greater soil aeration, drainage and rootable depth. It is also possible that CaSO₄ counteracts aluminum toxicity. We recommend that the effect of CaSO₄ on growth of potential biomass crops cultured on clayey soils be investigated.

Pathogens and Early Leaf Abscission:

The growth of black locust was also reduced (to an unknown extent) by early leaf abscission. Early leaf-fall occurred annually, and in some years nearly complete abscission was witnessed as early as late June. Abscission was earlier when spring and early summer rainfall was abundant. It occurred to an equal degree on every tree in the test -- it was not related to irrigation or nitrogen treatment or to genetic or microsite factors. These observations suggested that pathogens might be responsible. Roots and leaves were examined for pathogens by Dr. Ronald Roncadori of the University of Georgia Plant Pathology Department. No root pathogens were found that could be implicated in premature leaf drop. However, an anthracnose (*Colletotrichum gloeosporioides*), known to be very toxic was discovered on the leaves. Tests conducted by Dr. Roncadori and his student Cameron Whiting showed that this pathogen could produce the observed symptoms. The results of their work were reported Whiting et al 1994, which is reprinted in the Appendix.

Further work by Dr. Roncadori and Ms. Whiting has shown that *Colletotrichum gloeosporioides* may not be the only offending pathogen. Although black locust anthracnose infection was low this year, heavy leaf-fall was observed. Two other pathogens are currently under investigation. That pathogens are responsible for the premature leaf-fall appears likely because trees in the progeny test sprayed weekly with fungicides retained significantly more foliage than others.

If pathogens are conclusively implicated as the cause early abscission, the effect may be overcome with systemic fungicides, or more likely with resistance introduced through genetic transformation. There is no evidence in our progeny test that any degree of natural resistance is present. Before efforts to combat early leaf-fall are undertaken, however, its effect on yield should be ascertained.

NITROGEN FIXATION

Part of the interest in black locust as a biofuels crop is its ability to fix atmospheric nitrogen. Nitrogen fixation might reduce the need for nitrogen fertilization, improving cost effectiveness and energy input/output ratios, and perhaps foster acceptable yields on sites too poor for other species. However, nitrogen fixation is, itself, an energy consuming process that might occur at the expense of biomass yield. If so, nitrogen fertilization might be desirable in spite of nitrogen fixation capacity. But, the answer to that question may depend on the genotype utilized.

To answer these questions, we examined the relationship between growth, measured as dry weight accretion, soil nitrate concentration, and atmospheric N_2 fixation in seedlings of three open-pollinated families. In two separate experiments, growth, nitrogen fixation and its components, nodule biomass and nitrogenase activity were measured as soil nitrate concentration was varied. In the first experiment nitrogenase activity was measured by acetylene reduction. In the second, it was inferred from nitrogen uptake via fixation using labelled N^{15} . Results of this work were published in four articles, three of which are reproduced in the Appendix (Johnsen and Bongarten 1992, Johnsen and Bongarten 1991, Johnsen et al 1991). The most important results are enumerated here.

1. As expected from the work of other investigators, high levels of soil nitrate reduced nitrogen fixation by reducing nodule biomass and nitrogen fixation per unit nodule biomass.
2. Nodule biomass growth and nitrogenase activity were similar in seedlings grown without soil nitrate or with modest levels of soil nitrate. Previous reports of increases in these traits under modest levels of soil nitrate resulted from failure to allometrically adjust for effects of seedling size.
3. Growth of seedlings increased as soil nitrate level increased. This was attributable to lower assimilation costs associated with nitrogen derived from nitrate as opposed to atmospheric N_2 .
4. Unlike most plants, much of the nitrate reduction in black locust occurs in the leaves. This is believed to entail lower energy costs than nitrate reduction in roots, and may, in part, account for the lower costs of assimilating nitrate than N_2 .
5. As seedlings grew in size and nitrogen demand, soil nitrate concentration was not maintained with fertilizer applied at each watering. After two months, seedlings given the highest soil nitrate concentrations were obtaining most of their nitrogen via fixation. It is unlikely that soil nitrate concentrations can be maintained under field conditions at a level that will prohibit nitrogen fixation.
6. Unlike most species, root-shoot ratio was little influenced by soil nitrate concentration. Whereas most species increase root mass as soil nitrogen concentration declines, presumably to aid in nitrogen acquisition, black locust does not, but increases nitrogen uptake through fixation.
7. The three families examined, although differing in growth rate, showed similar relationships between growth, soil nitrate concentration and nitrogen fixation rate.

The above results suggest that application of nitrogen fertilizer should be beneficial to growth, although we have not determined if it is economically justifiable. Application at the beginning of the growing season appears most valuable because much of the nitrogen fixation machinery must be rebuilt each spring (most of the nodule die) limiting nitrogen availability at that time. Our results also suggest that the boost in growth from constant applications of nitrogen fertilizer will diminish as trees grow in size and nitrogen demand because diminishing fractions of the nitrogen uptaken will come from the fertilizer source. Interestingly, both predictions, increase growth with fertilization and diminishing effect of fertilization with increasing tree size, were realized in our field progeny tests.

VEGETATIVE PROPAGATION

Black locust may be vegetatively propagated by a variety of means including 1) root cuttings, 2) hardwood stem cuttings, 3) greenwood stem cuttings, 4) micropropagation with nodes and 5) tissue culture of leaf disks. Root cuttings have been used commercially, in Hungary, but cost limits their usage; fewer than 10% of plantings utilize the selected clones (Keresztesi, pers. comm.). Where feasible, stem cuttings offer the least costly means of vegetative propagation. We, therefore, focused our attention on developing a practical system for mass propagation by stem cuttings.

Early in the project we examined hardwood and greenwood cuttings. Rooting of hardwood cuttings was about 25%, whereas rooting of greenwood cuttings was over 50%. Furthermore, the hardwood cuttings showed persistent plagiotropism. As a result, subsequent work was limited to refining techniques of propagation by greenwood cuttings.

Several experiments were conducted, resulting in the following conclusions:

1. In some trials, rooting of greenwood cuttings exceeded 90%, although
2. Rooting can occur without hormone applications, but IBA supplied in talc or in ethanol improves rooting by about 50-20%. IBA is more effective than IAA or NAA.
3. Fungicide treatments are essential to prevent rotting. Thiram[®] gave better results than Daconyl[®] or Captan[®].
4. Rooting was improved in stem sections with some woody development. Shoot tips should be avoided.
5. Roots originate mainly from internodal tissue. There is no need to include a basal node.
6. Time to rooting varied by clone from 2 to 6 weeks. Generally, clones that rooted faster had greater rooting percentages, but late rooting clones could root as well when they could be kept fungus-free.
7. Cuttings may be taken several times during the growing season, without loss of rootability, by periodically forcing new growth by pruning.
8. Rooting is enhanced by using very porous media. Misting should be kept to a minimum without producing desiccation damage.
9. Plagiotropism can be reduced by taking cuttings from vigorous stump sprouts.
10. Most vigorous shoots possess proleptic shoots at each node. These are subject to high mortality (for unknown reasons) during rooting. As a result, many cuttings that have rooted eventually die for lack of a viable shoot meristem. Occasionally, however, adventitious shoots develop to replace the dead proleptic shoots.
11. Growth of stem cuttings following rooting is severely stunted.

At the present time, rooting of stem cuttings cannot be considered a viable means vegetative propagation. Although high rooting percentages can be achieved, survival and growth following rooting are less than satisfactory. These problems may be surmountable with further research, but the feasibility of alternative methods, especially micropropagation and root cuttings, should also be explored.

SOMATIC EMBRYOGENESIS AND GENE TRANSFER

The Merkle lab joined the project in 1991 with the overall goal of developing a system for inserting insect resistance genes into black locust trees. Expression of *Bacillus thuringiensis* endotoxin genes in the trees may offer protection against feeding by the coleopteran pests *Megacyllene robiniae* (locust borer) and *Odontata dorsalis* (locust leaf miner). However, before such genes could be tested in black locust, a transformation/regeneration system had to be developed for the species. At the time we joined the project, we possessed two tools that formed the basis for our approach. One of these tools was microprojectile-mediated gene transfer. We had already used this approach to produce transgenic yellow-poplar (*Liriodendron tulipifera*) trees by bombarding embryogenic yellow-poplar cultures. This approach made use of the fact that embryogenic yellow-poplar cultures could be maintained as rapidly proliferating suspensions of proembryogenic masses (PEMs). The PEMs were size-fractionated on sieves, collected on filter paper and bombarded with plasmid DNA (pBI121), which carried genes encoding β -glucuronidase (GUS) and neomycin phosphotransferase (NPTII). Kanamycin-resistant calli, which appeared after 6 weeks on selection medium, and somatic embryos and plantlets derived from them, were shown to carry the transferred DNA. Thus we believed that a key to successfully applying this approach to black locust was the development of rapidly-proliferating embryogenic suspension cultures. The other tool we possessed was a few embryogenic black locust cultures. These cultures had been initiated from immature black locust seeds that had been collected within 4 weeks following anthesis, and cultured on MS medium supplemented with 2,4-D for 3-7 days prior to being transferred to hormone-free medium MS medium. However, unlike the yellow-poplar cultures, the black locust cultures proliferated slowly by repetitive embryogenesis from the radicles of older embryos. They could not be maintained as rapidly-growing suspension cultures of PEMs and thus did not appear to make good target tissue for microprojectile bombardment. These cultures also had the problem of producing somatic embryos that germinated precociously, which not only produced a weak, spindly plantlet, but halted repetitive embryogenesis. Just prior to joining the project, we had found that transferring these cultures to a layer of filter paper on top of semisolid MS medium induced production of PEMs similar to those in embryogenic yellow-poplar cultures, although we were not sure we could maintain proliferation of the PEMs. Thus, we had two tools which we hoped to apply to genetic transformation of black locust. On this basis, our objectives when we began the project in 1991 were:

- (1) To establish culture conditions that will maintain embryogenic black locust cultures as PEMs, making them amenable for the initiation of embryogenic suspension cultures.
- (2) To transfer foreign DNA containing kanamycin resistance and GUS marker genes into embryogenic black locust cells by using PEMs as targets for microprojectile bombardment.
- (3) To screen bombarded cultures for transformed colonies using kanamycin resistance, and to assay putative transformants for presence and expression of the inserted DNA.
- (4) To regenerate transformed black locust somatic embryos and plantlets and confirm their transformed genotypes.

As we became more involved with black locust tissue culture and gene transfer, the need to explore other aspects of these systems became apparent. Because we had only a single surviving embryogenic black locust line, one problem that had to be solved was development of a reliable protocol for the initiation of new embryogenic cultures. Other challenges were development of a delivery system for black locust somatic embryos and establishment of an alternative in vitro regeneration system for black locust that might be useful for gene transfer, in case somatic embryogenesis did not prove a useful route. Thus, three more objectives were added:

- (5) To develop a reliable protocol for initiation of embryogenic black locust cultures.

- (6) To test encapsulated and nonencapsulated black locust somatic embryos as artificial seeds.
- (7) To establish a high-frequency adventitious bud regeneration system for black locust as a possible alternative route for gene transfer.

Summary of Methods and Results

Here, we summarize the progress that was made on each of the objectives during the period 1991-1993. More detailed descriptions of the methods and results may be found in the papers cited in the summaries below. They are reprinted here, following the summaries.

Objective 1. Development of embryogenic suspension cultures.

PEMs grew very slowly if maintained on filter paper overlaid on hormone-free, semisolid MS medium. Therefore, a new medium, FM, was tested for maintenance of more rapid proliferation of PEMs. This medium was based on a medium developed by Finer and Nagasawa (1988) for maintenance of embryogenic soybean suspension cultures. Our modified version of this medium contained Finer and Nagasawa's major salts, MS minor salts, Gresshoff and Doy's (1972) vitamins, 15 mM glutamine, 3 mg/l 2,4-D, 0.25 mg/l BAP and 6% sucrose. PEMs continued to proliferate on this medium without producing later stages of embryo development. Furthermore, PEMs could be grown as suspensions in a liquid version of FM. PEMs transferred to a hormone-free version of liquid FM produced large numbers of somatic embryos directly in suspension. Establishment of embryogenic black locust suspension cultures is detailed in Merkle (1992).

Objectives 2,3 and 4. Microprojectile-mediated gene transfer to PEMs, antibiotic screening of bombarded cultures, assays for stable integration of transferred DNA and regeneration of transformed somatic embryos and plantlets.

Our results from microprojectile-mediated gene transfer experiments with embryogenic black locust cultures were reported in a summary report dated September 15, 1992 and in Arrillaga and Merkle (in press). These reports detail the results from experiments in which plasmid DNA was delivered into suspension cultured proembryogenic masses (PEMs) of black locust line BL2-1C via microprojectile bombardment using a DuPont PDS1000/He particle delivery device. We tested the following variables for their effect on transient GUS expression in the bombarded cells: (1) size of bombarded black locust cell clusters, (2) gas pressure used to accelerate the macrocarrier, (3) gap distance between the rupture disk and the macrocarrier, and (4) the promoter used to control expression of the GUS gene. To test promoters, two plasmids, pBI121 and pBI2S21 were used. Both plasmids encode the selectable marker neomycin phosphotransferase (NPTII) and the reporter gene β -glucuronidase (GUS), but in pBI121, the GUS gene is fused with the CaMV35S promoter, while in pBI2S21, the GUS gene is fused to the promoter from the *Arabidopsis* actin gene *Ac2*. The plasmid pBI2S21 was given to us by Dr. Richard Meagher of the UGA Genetics department. Before these experiments were begun, we tested different antibiotics as potential selection agents, including kanamycin, geneticin and hygromycin. Relatively high levels of kanamycin (>200 μ g/ml) were required to suppress growth of black locust PEMs, while much lower levels of geneticin or hygromycin were required. Since the plasmids to which we had access carried a gene for NPTII, which confers resistance to both kanamycin and geneticin, we decided to use geneticin at 15 μ g/ml as the selection agent in our experiments.

We found that the greatest number of GUS-positive foci were obtained using cell clusters smaller than 380 μ m, a pressure of 1100 psi, a gap distance of 1/4 inch, and the *Ac2* promoter. Although these bombardment conditions and promoter gave us significantly higher levels of transient expression than the others tested, we failed to find evidence of stable integration of the transferred

DNA. Bombarded cell clumps plated on geneticin-supplemented selection medium either 7 or 14 days following bombardment produced colonies of PEMs which appeared to be resistant to the antibiotic. However, none of the resistant colonies tested showed evidence of GUS expression, indicating that they probably represented cells which escaped selection rather than stably transformed cells.

We employed the following strategies in our experiments in 1993: (1) We continued to test resistant colonies of PEMs that appeared in our cultures under geneticin selection for GUS activity; (2) We generated enough suspension cultured material from each of our new embryogenic black locust cultures (see work under Objective 5) to use them for microprojectile-mediated gene transfer experiments. The additional embryogenic lines gave us the opportunity to test our transformation protocol with younger, genetically diverse material; (3) We obtained access to a new plasmid (pTRA140) carrying genes for GUS and for resistance to hygromycin. Since previous experiments had shown that 20 μ g/ml of hygromycin inhibited growth of black locust PEMs, this level of the antibiotic was used for selection. Microprojectile bombardment experiments employing the new plasmid were conducted, but transient expression levels in bombarded cells were very low and no hygromycin-resistant colonies were produced; (4) A group of cultures that displayed long-term (several months) resistance to geneticin was tested for expression of the NPTII gene using an ELISA kit from 5 Prime-3 Prime, Inc. Two of the putatively transformed lines displayed NPTII expression that was above background, but weak. (5) DNA was extracted from PEMs of the same long-term resistant cultures and attempts were made to amplify fragments of the GUS gene using polymerase chain reaction (PCR). No amplified products were detected. (6) At the time of this report, somatic embryos had been generated from the long-term resistant cultures and DNA was extracted from these embryos for Southern hybridization with probes made from both the NPTII and GUS genes.

Objective 5. Development of a reliable protocol for initiation of embryogenic black locust cultures.

During spring and summer, 1992, an experiment was conducted to define the optimal conditions for initiation of embryogenic black locust cultures. Developing black locust fruits were collected from 7 different black locust trees on a weekly basis from one week post-anthesis to 7 weeks post-anthesis. Immature seeds or excised embryos were cultured on a primary medium with three different combinations of 2,4-D and BA for 15 days, after which they were transferred to one of four different secondary media. All media were based on FM (Finer and Nagasawa 1988). Best results (average of 12% embryogenic cultures) were obtained when seeds collected 2-3 weeks post-anthesis were cultured for 3 weeks on FM containing 45-90 μ M 2,4-D and 2.2 μ M BAP, followed by transfer to the same medium without growth regulators. Some embryos excised from seeds later than 5 weeks subsequent to anthesis produced multiple embryos, but these were always fused and misshapen, and failed to continue to proliferate as repetitive embryogenic cultures. There was also some evidence that seeds from some mother trees had a higher potential to produce embryogenic cultures than seeds of other trees. At least five of the new embryogenic cultures produced PEMs which were used to initiate embryogenic suspension cultures used in microprojectile-mediated gene transfer experiments. Details of the culture initiation experiment can be found in Arrillaga et al. (1994).

Objective 6. Testing of black locust somatic embryos as artificial seeds.

One potential advantage of employing embryogenic cultures for propagation of transgenic trees is their potential for somatic embryos to be deployed directly into the greenhouse or even the field as artificial seeds. Therefore, we initiated an experiment to test the potential of naked and encapsulated black locust somatic embryos to survive cold storage and convert to plantlets. Globular stage black locust somatic embryos were obtained by transferring approximately 0.5 g of PEMs from liquid FM supplemented with 3 mg/l 2,4-D to hormone-free liquid FM. Cotyledonary stage embryos were obtained when the globular stage embryos were plated on the same medium solidified with agar. For encapsulation, cotyledonary embryos were placed in a beaker of 2% sodium alginate solution. Then drops of alginate solution containing individual embryos were pipetted into a beaker

if 50 mM calcium chloride solution, forming a calcium alginate bead around each embryo. Naked and encapsulated embryos were stored on moist filter paper at 4° C for 0, 15, 45, or 100 days, after which they were tested for germination on half-strength MS medium. With no cold treatment, 71% of the naked embryos and 41% of the encapsulated embryos converted into plantlets. Fifteen days of cold treatment increased conversion rates up to 95% for naked embryos and 80% for encapsulated embryos. Cold treatments longer than 15 days resulted in lower conversion percentages. Recovered plantlets were acclimatized and grown in the greenhouse. In another experiment, naked and encapsulated black locust somatic embryos were tested for their ability to germinate directly on potting mix in GA7 vessels (Magenta Corp.). As was the case with conversion on tissue culture medium, a higher percentage of naked embryos (18%) converted to plantlets than encapsulated embryos (7%). These experiments are detailed in Arrillaga et al. (1994).

Objective 7. Establishment of a high-frequency adventitious bud regeneration system for black locust.

Although most of our efforts towards development of a system for generation of transgenic black locust trees have concentrated on employing somatic embryogenesis, another potential route for gene transfer would be via transformation of cells in leaves or cotyledons, either via *Agrobacterium*-Ti plasmid-mediated gene transfer or microprojectile bombardment. Therefore, some experiments were established to investigate the potential for regenerating black locust plantlets via leaf or cotyledon explants. Direct adventitious shoots were induced from cotyledon explants on Woody Plant Medium (WPM; Lloyd and McCown 1980) containing 22.2 μ M BA and 0.4 μ M 2,4-D. Shoots developed and new shoots were induced when the explants were transferred to a medium without growth regulators. The effect of dark incubation on shoot regeneration from cotyledons indicated that 15 days of darkness resulted in high regeneration frequency (91.7%). Adventitious shoot formation was also induced from sections of in vitro-derived leaves cultured in the dark on Murashige and Skoog's (MS; 1962) medium supplemented with 4.4 μ M BA and 24.6 μ M IBA. A shoot regeneration frequency of 89% was obtained when explants were subcultured on a medium containing 4.4 μ M BA and 0.5 μ M IAA. Shoots were rooted on Schenk and Hildebrandt's (1972) medium with or without IBA. Plantlets were acclimatized and grown in the greenhouse. These experiments are detailed in Arrillaga and Merkle (1993).

CONCLUDING REMARKS

During this project we have learned a great deal about the biology, and particularly the genetics of black locust. It is now possible to design breeding programs that can effectively improve biomass yield; superior individuals have been identified which could form the nucleus of an improved breeding population. As a result, productivity of black locust in short-rotation, intensive culture could reasonably be doubled in a generation or two of selective breeding (due in large part to the great variation that is inherently present). It is unlikely, however, that black locust can achieve the desired goal of $20 \text{ mg ha}^{-1} \text{ yr}^{-1}$ on eroded upland Piedmont sites. It is doubtful that any woody species can.

Nevertheless, black locust exhibits exceptionally rapid juvenile growth. Its downfall on agriculturally degraded Piedmont lands is attributable to the heavy clay soils which limit rooting, slowing growth by the third year. Other results indicate that the potential of black locust will be best expressed on soils of looser texture. It would be a mistake to judge black locust on its performance on sites to which it is ill-suited and which are notoriously low in productive capacity. There is ample evidence to support continued develop of this species as a biofuels crop on appropriate sites.

For effective utilization of black locust, development efforts should focus on four items. First, the range of sites on which black locust will maintain sustained growth should be determined. Second, efficient means for vegetatively propagating black locust should be further enhanced. Third, a reasoned ideotype for black locust as a fuel crop, melding biological and economic considerations, should be constructed. Finally, methods for controlling insects and diseases must be generated.

In the long-run, the final item may be the most important. If black locust has an Achilles' heel, it is susceptibility to pathogens and insects. The full potential of black locust will be realized only when these are controlled. At the present time, the most promising avenue for pest control appears to be the biotechnology highway. Continued investment in this technology could pay large dividends in future growth.

LITERATURE CITED

- Arrillaga, I., and S.A. Merkle. Transient gene expression in embryogenic black locust cultures following microprojectile bombardment. *In* Proceedings of the IUFRO Workshop on Biotechnology of Trees (M.R. Ahuja, ed.). Revista Investigacion Agraria. Sistemas y Recursos Forestales (in press).
- Arrillaga, I., and S.A. Merkle. 1993. Plant regeneration from in vitro culture of cotyledon and leaf explants of black locust. *HortScience* 28:942-945.
- Arrillaga, I., J.J. Tobolski, and S.A. Merkle. 1994. Advances in somatic embryogenesis and plant production in black locust (*Robinia pseudoacacia* L.) *Plant Cell Reports* (in press).
- Bloese, P., J. W. Hanover, and B. C. Bongarten. 1992. Inheritance of juvenile traits and predicted gains from selection in black locust progeny tests in Michigan and Georgia. *In*: Black Locust: Biology, Culture and Utilization (J. W. Hanover, K. Miller and S. Plesko eds.). Michigan State Univ., E. Lansing. pp. 97-107.
- Bongarten, B. C. 1992. Genetic variation in black locust in its native range. *In*: Black Locust: Biology, Culture and Utilization (J. W. Hanover, K. Miller and S. Plesko, eds.). Michigan State Univ., E. Lansing. pp. 78-97.
- Bongarten, B. C. and J. F. Dowd. 1987. Regression and spline methods for removing environmental variance in progeny tests. *In* Proc. 19th South. Forest Tree Impr. Conf. (C. R. McKinley and W. A. Lowe, eds.), College Station, TX. June 16-18, 1987. pp. 312-319.
- Bongarten, B. C., D. A. Huber and D. K. Apsley. 1992. Environmental and genetic influences on short-rotation biomass production of black locust (*Robinia pseudoacacia* L.) in the Georgia Piedmont. *For. Ecol. Manag.* 55:315-331.
- Bongarten, B. C., S. A. Merkle and J. W. Hanover. 1991. Genetically improved black locust for biomass production in short-rotation plantations. *In* Energy from Biomass and Wastes XV (D. L. Klass, ed.), Institute of Gas Technology, Chicago, IL. p. 391-409.
- Finer, J.J. and A. Nagasawa. 1988. Development of an embryogenic suspension culture of soybean [*Glycine max* (L.) Merrill.]. *Plant Cell Tissue Organ Cult.* 15:125-136.
- Genys, J.B. and D.M. Harman. 1990. Racial diversity of black locust in growth rate and in susceptibility to the locust twig borer
- Jefferson, R.A., T.A. Kavanaugh, and M.W. Bevan. 1987. GUS fusions: β -glucuronidase as a versatile gene fusion marker in higher plants. *EMBO J.* 6:3901-3907.
- Johnsen, K. H. and B. C. Bongarten. 1992. Effects of nitrate on nitrogen fixation and growth of *Robinia pseudoacacia* seedlings: A functional growth analysis approach using ^{15}N . *Physiol. Plant.* 85:77-84.
- Johnsen, K. H. and B. C. Bongarten. 1991. Effects of nitrate on acetylene reduction and growth of seedlings from three open-pollinated families of *Robinia pseudoacacia*. *Tree Physiol.* 9:507-522.
- Johnsen, K. H., B. C. Bongarten and L. R. Boring. 1991. Effects of nitrate on *in vivo* nitrate reductase activity of seedlings from three open-pollinated families of *Robinia pseudoacacia*. *Tree Physiol.* 8:381-389.

- Lloyd, G., and B.H. McCown. 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Comb. Proc. Intl. Plant Prop. Soc. 30:421-427.
- Kennedy, J.M. 1983. Geographic variation in black locust (*Robinia pseudoacacia* L.) M.Sc. Thesis, The University of Georgia, Athens, GA, USA, 66 pp.
- Mebrahtu, T. and J.W. Hanover. 1989. Heritability and expected gain estimates for traits of black locust in Michigan. *Silvae Genet.*, 38:125-130.
- Merkle, S.A. 1992. Somatic embryogenesis in black locust. In *Black Locust: Biology, Culture and Utilization* (J.W. Hanover, K. Miller, and S. Plesko, eds.). Michigan State University Department of Forestry. pp. 136-146.
- Merkle, S.A., and A.T. Wiecko. 1989. Regeneration of *Robinia pseudoacacia* via somatic embryogenesis. *Can. J. For. Res.* 19:285-288.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* 15:473-497.
- Schenk, R.V., and A.L. Hildebrandt. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot.* 50:199-204.
- Sumner, M.E., W.P. Miller, D.E. Radcliffe, and W.I. Segars. 1989. Principles of gypsum use as a soil amendment. Univ. Georgia Coop. Ext. Serv. Rep. MP-37. University of Georgia, Athens, GA, 8 pp.
- Surles, S. E., J. L. Hamrick and B. C. Bongarten. 1990. Mating systems in open-pollinated families of black locust (*Robinia pseudoacacia*). *Silvae Genet.* 39:35-40.
- Surles, S. E., J. Arnold, A. Schnabel, J. L. Hamrick and B. C. Bongarten. 1990. Genetic relatedness in open-pollinated families of two leguminous tree species, *Robinia pseudoacacia* L. and *Gleditsia triacanthos* L. *Theor. Appl. Genet.* 80:49-56.
- Surles, S. E., J. L. Hamrick and B. C. Bongarten. 1989. Allozyme variation in black locust (*Robinia pseudoacacia*). *Can. J. For. Res.* 19:471-479.
- Whiting, E. C., R. Roncadori and B. C. Bongarten. 1994. First report of black locust anthracnose caused by *Colletotrichum gloeosporioides* in Georgia and South Carolina. *Plant Dis.* 78:529.

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PROJECT PUBLICATIONS

Journal Articles and Book Chapters

- Arrillaga, I. and S.A. Merkle. Transient gene expression in embryogenic black locust cultures following microprojectile bombardment. In Proceedings of the IUFRO Workshop on Biotechnology of Trees (M.R. Ahuja, ed.). Revista Investigacion Agraria. Sistemas y Recursos Forestales (in press).
- Arrillaga, I., and S.A. Merkle. 1993. Plant regeneration from in vitro culture of cotyledon and leaf explants of black locust. *HortScience* 28:942-945.
- Arrillaga, I., J.J. Tobolski, and S.A. Merkle. 1994. Advances in somatic embryogenesis and plant production in black locust (*Robinia pseudoacacia* L.) *Plant Cell Reports* (in press).
- Bloese, P., J. W. Hanover, and B. C. Bongarten. 1992. Inheritance of juvenile traits and predicted gains from selection in black locust progeny tests in Michigan and Georgia. In: Black Locust: Biology, Culture and Utilization (J. W. Hanover, K. Miller and S. Plesko eds.). Michigan State Univ., E. Lansing. pp. 97-107.
- Bongarten, B. C. 1993. The itinerant tree. *The World & I.* 10:202-207.
- Bongarten, B. C. 1992. Genetic variation in black locust in its native range. In: Black Locust: Biology, Culture and Utilization (J. W. Hanover, K. Miller and S. Plesko, eds.). Michigan State Univ., E. Lansing. pp. 78-97.
- Bongarten, B. C. and J. F. Dowd. 1987. Regression and spline methods for removing environmental variance in progeny tests. In Proc. 19th South. Forest Tree Impr. Conf. (C. R. McKinley and W. A. Lowe, eds.), College Station, TX. June 16-18, 1987. pp. 312-319.
- Bongarten, B. C. and Ji, L. 1994. Selection of sweetgum and sycamore from provenance-progeny tests. In Proc. 23rd South. Forest Tree Impr. Conf. (W. McConnell and T. LaFarge, eds.), Atlanta, GA. June 14-17, 1993. 124-131.
- Bongarten, B. C., D. A. Huber and D. K. Apsley. 1992. Environmental and genetic influences on short-rotation biomass production of black locust (*Robinia pseudoacacia* L.) in the Georgia Piedmont. *For. Ecol. Manag.* 55:315-331.
- Bongarten, B. C., S. A. Merkle and J. W. Hanover. 1991. Genetically improved black locust for biomass production in short-rotation plantations. In Energy from Biomass and Wastes XV (D. L. Klass, ed.), Institute of Gas Technology, Chicago, IL. p. 391-409.
- Johnsen, K. H. and D. K. Apsley. 1991. A simple method to measure acetylene reduction of intact, nodulated tree seedlings. *Tree Physiol.* 9:501-506.
- Johnsen, K. H. and B. C. Bongarten. 1992. Effects of nitrate on nitrogen fixation and growth of *Robinia pseudoacacia* seedlings: A functional growth analysis approach using ¹⁵N. *Physiol. Plant.* 85:77-84.
- Johnsen, K. H. and B. C. Bongarten. 1991. Effects of nitrate on acetylene reduction and growth of seedlings from three open-pollinated families of *Robinia pseudoacacia*. *Tree Physiol.* 9:507-522.

- Johnsen, K. H., B. C. Bongarten and L. R. Boring. 1991. Effects of nitrate on *in vivo* nitrate reductase activity of seedlings from three open-pollinated families of *Robinia pseudoacacia*. *Tree Physiol.* 8:381-389.
- Land, S.B., B.C. Bongarten and J.R. Toliver. 1987. Genetic parameters and selection indices from provenance/progeny tests. In Statistical considerations in genetic testing of forest trees. Proc. of the 1986 Wksp. of SREIG-40 (D.L. Rockwood, ed.), South. Coop. Series Bull. No. 324. pp. 59-74.
- Merkle, S.A. and R.N. Trigiano. 1994. *In vitro* propagation of hardwoods. In Applications of Vegetative Propagation in Forestry, Proceedings of the 1992 SREIG-40 Biennial Symposium on Forest Genetics, July 8-10, 1992, Huntsville. p. 23-38.
- Merkle, S.A. 1992. Somatic embryogenesis in black locust. In Black Locust: Biology, Culture and Utilization (J.W. Hanover, K. Miller, and S. Plesko, eds.). Michigan State University Department of Forestry. pp. 136-146.
- Merkle, S.A., W.A. Parrot and B.S. Flinn. 1994. Morphological aspects of somatic embryogenesis. In *Vitro Embryogenesis in Plants* (T.A. Thorpe, ed.). CRC Press, Inc., Boca Raton (in press).
- Merkle, S.A., and A.T. Wiecko. 1989. Regeneration of *Robinia pseudoacacia* via somatic embryogenesis. *Can. J. For. Res.* 19:285-288.
- Surles, S. E., J. L. Hamrick and B. C. Bongarten. 1990. Mating systems in open-pollinated families of black locust (*Robinia pseudoacacia*). *Silvae Genet.* 39:35-40.
- Surles, S. E., J. Arnold, A. Schnabel, J. L. Hamrick and B. C. Bongarten. 1990. Genetic relatedness in open-pollinated families of two leguminous tree species, *Robinia pseudoacacia* L. and *Gleditsia triacanthos* L. *Theor. Appl. Genet.* 80:49-56.
- Surles, S. E., J. L. Hamrick and B. C. Bongarten. 1989. Allozyme variation in black locust (*Robinia pseudoacacia*). *Can. J. For. Res.* 19:471-479.
- Trigiano, R.N., R.L. Geneve, S.A. Merkle, and J.E. Preece. 1992. Tissue and cell cultures of woody legumes. *Horticulture Reviews*, Vol. 14 (J. Janick, ed.). John Wiley & Sons, Inc., NY. pp. 265-332.
- Whiting, E. C., R. Roncadori and B. C. Bongarten. 1994. First report of black locust anthracnose caused by *Colletotrichum gloeosporioides* in Georgia and South Carolina. *Plant Dis.* 78:529.

Presentations and Abstracts

- Arrillaga, I., J.J. Tobolski, and S.A. Merkle. 1994. Effect of cold treatment on the conversion of black locust somatic embryos. Proc. 22nd South. For. Tree Impr. Conf., June 14-17, 1993, Atlanta. p. 474.
- Bongarten, B. C. and C. S. Chang. 1994. Genetic structure of black locust in native stands. 13th North Amer. For. Biol. Wksp. Baton Rouge, LA, June 14-16. p. 18.
- Bongarten, B. C. and K. H. Johnsen. 1992. Allometry of shoot-root ratio in tree seedlings. 77th Ecol. Soc. Amer. Meeting. Honolulu, HI, Aug. 9-13. Bull. Ecol. Soc. Amer. 73:116.

- Bongarten, B. C. 1991. Genetic structure of black locust in its native range. International Conf. on Black Locust: Biology, Culture, Utilization. East Lansing, MI, June 17-21.
- Bongarten, B. C. 1991. Meeting summary and plans for future research. International Conf. on Black Locust: Biology, Culture, Utilization. East Lansing, MI, June 17-21.
- Bongarten, B. C. 1991. Yields from black locust (*Robinia pseudoacacia*) selected for biomass production. Ninth South. Biomass Energy Conf. Baton Rouge, January 8-10.
- Bongarten, B. C. 1991. Belowground root research: root microbial interactions. Biofuels Energy Conference, Davis, CA, Sept. 16-20.
- Bongarten, B. C. 1989. Selection of black locust for biomass production. Terrestrial Energy Crops Program Conf. & Wksp. Washington, DC, July 18-21.
- Bongarten, B. C. 1988. Breeding and selection strategies: *Robinia*. Contractors' Wksp, Short Rotation Woody Crops Program and IEA Wksp. on Plantation Economics and Coppice. Tacoma, WA, Aug. 1-5.
- Bongarten, B. C. 1987. How physiological research benefits plantation establishment: tree improvement. In Proc. Soc. Amer. Foresters Convention, Minneapolis, MN, October 18-21. pp.
- Bongarten, B. C. 1987. Black locust research. Subcontractors' Wksp, Short Rotation Woody Crops Program. Oak Ridge, TN, Aug. 4-6.
- Bongarten, B. C. 1986. Survey of genetic variation in potential Energy plantation species for the South. In Proc. Fourth South. Biomass Energy Conf., Nov. 4-6. (J. W. Goodrum, ed.).
- Bongarten, B. C., S. A. Merkle and J. W. Hanover. 1991. Genetically improved black locust for biomass production in short-rotation plantations. In Energy from Biomass and Wastes XV, Washington, D.C., March 21-24.
- Boring, L.R. and D.K. Apsley. Measurement of nitrogen fixation in *Robinia pseudoacacia* using ^{15}N isotope dilution techniques. Annual meeting of the Ecological Society of America, Davis, CA. August.
- Boring, L.R. and D.K. Apsley. ^{15}N isotope dilution techniques adapted to measure N_2 fixation in black locust. Annual DOE cooperator's meeting, Tacoma, WA. July.
- Johnsen, K. H. and B. C. Bongarten. 1990. Effects of soil nitrate on nitrogen fixation and nitrogen productivity of black locust (*Robinia pseudoacacia* L.) seedlings: A functional growth analysis approach using ^{15}N . 11th North Amer. For. Biol. Wksp. Athens, GA, June 13-15.
- Johnsen, K. H. and B. C. Bongarten. 1990. Effects of nitrate on nitrogen fixation and growth of *Robinia pseudoacacia* seedlings. 72nd Ann. Meeting Ecol. Soc. Amer. Snowbird, UT, Aug. 1-4.
- Johnsen, K. H., B. C. Bongarten, L. R. Boring and R. O. Teskey. 1989. Short-term effects of nitrate on nitrogen metabolism of black locust seedlings. 71st Ann. Meeting. Ecol. Soc. Amer. Toronto, Ont., July 31- Aug. 3.
- Merkle, S. A. 1991. Somatic embryogenesis of black locust. International Conference on Black Locust: Biology, Culture and Utilization. East, Lansing, MI, June 17-21.

- Merkle, S. A., H. D. Wilde, A. T. Groover, D. T. Carraway and B. A. Watson. 1991. Embryogenic suspension cultures for genetic transformation and mass propagation of hardwood forest trees. In Abstracts of papers Presented at the International Symposium on Applications of Biotechnology to Tree Culture, Protection and Utilization. USDA Forest Service Northeast For. Expt. Sta. Gen. Tech. Rep. NE-152. p. 116.
- Tobolski, J.J., I. Arrillaga and S.A. Merkle. 1992. Encapsulation of black locust somatic embryos. Proc. 12th North Amer. For. Biol. Wksp. Aug. 17-20, 1992, Sault Ste. Marie, Ont. p. 201.

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