ANNUAL PROGRAM REVIEW

FOREST BIOLOGY

March 21-22, 1995

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Institute of Paper Science and Technology
500 10th Street, N.W.
Atlanta, GA 30318
(404) 853-9500

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S O F T W O O D S

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

Status Report for
Project F010

Gerald Pullman
John Cairney
Shujun Chang

March 21-22, 1995

Institute of Paper Science and Technology
500 10th Street, N.W.
Atlanta, GA 30318
(404) 853-9500

TECHNICAL PROGRAM REVIEW
FY 94-95

Project Title:	MASS CLONAL PROPAGATION OF IMPROVED CONIFERS
Project Code:	SFTWD
Project Number:	F-010
Division:	Chemical and Biological Sciences
Project Staff:	Gerald Pullman, John Cairney, Shujun Chang, Barbara Johns, Shannon Johnson, Yolanda Powell, Camille Stephens
FY 94-95 Budget:	$408,000

PROGRAM OBJECTIVE:

Develop reliable cell and tissue culture systems for mass clonal propagation of genetically improved softwoods.

SUMMARY OF RESULTS:

The Institute's first crop of loblolly pine somatic seedlings has been produced. Approximately 50 somatic embryos from a single genotype have germinated and been acclimated to growth in soil. Somatic embryos from two additional genotypes have germinated to produce a few plants with shoot growth but no root growth yet.

Data for the osmotic environment of developing loblolly pine zygotic embryos has been analyzed and summarized. Two growing seasons (Summer 1994 and 1995) of osmotic measurements for the whole ovule (female gametophyte and embryo) provide us with a striking picture of the osmotic environment during embryo development. The findings provide keys to future experimentation and help to explain why maltose provides a superior development environment over sucrose. Measurements of ovule, embryo, and female gametophyte fresh and dry weights, % water content and embryo stage during development provide additional information for comparison of somatic and zygotic embryos.

An O-methyl transferase gene, presumed to be involved in lignin synthesis, has been cloned into a gene transfer vector, *Agrobacterium tumefaciens*. Transformation experiments with this gene are now in progress and await plant selection and screening.

Genomic clones of a proteinase inhibitor have been isolated and cloned. By using these as "probes" DNA sequences that regulate the proteinase inhibitor genes have been isolated. An understanding of the function of these regulator sequences will allow increased control and expression of genes of commercial interest when moved into target plants.

Chemical analysis of the activated charcoal-containing initiation medium has identified two metal ions which are heavily adsorbed by the activated charcoal. Approximately 90% of the media copper ions and 50% of the media zinc ions are made unavailable due to adsorption by activated charcoal. The loss of these two essential ions may be inhibiting somatic embryo growth just as the initiation process begins. Both analysis data and tests with the somatic model system tend to support the above hypothesis.

Growth kinetics of three loblolly pine liquid-grown cultures show similar growth patterns over an extended subculture cycle. We expected that this information will help us to improve the liquid culture system in the future and thus produce higher quality early-stage embryos.

Natural Zygotic Embryogenesis - IPST Ph. D. student Renee Kapik has completed his work on Abscisic acid analysis in naturally developing loblolly pine embryos. Time course data for three 1/2 sib families show similar patterns of ABA increasing to a peak and then decreasing through time. This work provides a model for the development of somatic embryos. In addition, the method of ABA analysis can be applied to somatic embryos in order to target ABA levels present in zygotic embryos.

The molecular biology is laboratories are "up and running". The necessary techniques of nucleic acid extraction, gene cloning, Reverse Transcription-PCR, and Northern and Southern analysis are all operational and functioning well in the laboratory. A major effort was spent in the last year obtaining the necessary radioactive chemical licenses. Necessary model systems of plant transformation and regeneration with tobacco and arabidopsis are operational.

Several member companies are interested in licensing the IPST Maltose patent. A special thanks go to Ron Dinus for his persistence in seeing this patent to issuance. It is expected that this patent will provide significant income to the Forest Biology Group in the future.

Eight research grant proposals (see the list at the end of this PAC Report), representing over $1,000,000 for IPST, were submitted to various research agencies in order to supplement the level of funding in the Forest Biology Group. Significant efforts by all faculty members have been applied to building research teams within and outside of the institute.

Softwood Project Specific Goals and Milestones

1994 / 1995

Somatic Embryogenesis

Initiation of Embryogenic Suspensor Mass

In 1993 we increased the extrusion rate by the use of an activated charcoal / high hormone initiation medium. Observations show that extrusion often occurs followed by the production of a few somatic embryos but these rarely continued growth. This year we will focus on determining the reason for the lack of continued growth in the presence of the activated charcoal initiation medium. We plan to :

1. Determine activated charcoal adsorption capacity of media components through analysis of macro and micro elements. (94.3)
2. Use the somatic initiation model to determine effect of increasing individual macro and micro nutrients. (94.4)
3. Test hypotheses developed from findings from goal 1 using zygotic initiation. (94.4)
4. Develop a kinetics model for activated charcoal adsorption of hormones (2,4-D) in initiation medium. (MS Student Project). (95.2)

This work will be carried out by the Forest Biology Technical Staff (Barbara Johns, Shannon Johnson, Yolanda Powell), Research Services Division, and MS graduate student Andrew Toering with assistance from Dr. Jerry Pullman.

Maintenance of Liquid Grown Cultures

We hope to improve the quality of early-stage embryos grown in liquid culture and the responsiveness of genotypes by better understanding the culture process itself and by understanding the nutritional, hormonal, and environmental parameters driving natural embryo development.

1. Improve understanding of liquid culture process. Determine growth kinetics of embryos in suspension culture. (94.3)
2. Explore hypotheses for advancement of embryo stage and improvement of responding genotypes in liquid suspension culture. (95.2)

This work will be carried out by the Forest Biology Technical Staff (Barbara Johns, Shannon Johnson, Yolanda Powell) with the assistance of Dr. Jerry Pullman.

Embryo Maturation

Improvement of embryo quality is necessary to move this technology towards commercialization. Work will focus on improving embryo maturation through attempts to mimic the nutritional, hormonal, and environmental conditions found during natural embryo development.

1. Use ABA ELISA analysis system developed by Ph.D. Student work.
    a. Characterize ABA levels present in somatic embryos. (95.1)
    b. Compare levels present in zygotic and somatic embryos. (95.1)

    c. Modify ABA in media to grow somatic embryos matching target levels in zygotic embryos. (95.2)
2. Use osmotic measurements from developing zygotic embryos to develop hypotheses for improvement of embryo quality. Test hypotheses by modification of osmotic sequence in maturation medium. (95.2)
3. Compare mineral composition of mature zygotic embryos and most advanced somatic embryos from best protocol.
    a. Analyze micro and macro elements present in mature zygotic female gametophytes and embryos and most advanced somatic embryos. ((95.1)
    b. Compare analyses for statistically significant differences. (95.1)
    c. Adjust medium to produce somatic embryos that contain elemental concentrations that fall within the natural range of zygotic embryos. (95.2)

This work will be carried out by the Forest Biology Technical Staff (Barbara Johns, Shannon Johnson, Yolanda Powell) and Research Services Division with the assistance of Dr. Jerry Pullman.

Germination and Establishment in Soil

1. Grow first loblolly pine somatic seedling. (95.2)
2. Determine effect of desiccation and stratification treatments on somatic embryo germination. (95.2)

This work will be carried out by the Forest Biology Technical Staff (Barbara Johns, Shannon Johnson, Yolanda Powell) with the assistance of Dr. Jerry Pullman.

Improve Understanding of Zygotic Embryo Development

Ideally, somatic embryos should resemble zygotic embryos at all levels starting with gene expression and ending with the accumulation of storage products. This year we expect to complete the abscisic acid analysis of developing zygotic embryos, begin analysis of IAA (an important hormone in the auxin group), and begin analysis of the osmotic environment during embryo development. In addition, we hope to begin an exploratory program with researchers at the Georgia Institute of Technology on gene expression during embryo development.

1. Complete ABA analysis work (Ph.D. Student). (95.2)
2. Begin development of IAA ELISA analysis technique (MS Student). (95.2)
3. Measure osmotic levels during embryo development. (95.1)
4. Begin characterization of components responsible for the osmotic environment. Start by determining levels of free amino acids during embryo development. (95.1)
5. Begin exploration of gene expression in staged zygotic and somatic embryos including genes responsible for storage product accumulation. Use stage specific markers to assess and improve maturation and quality of somatic embryos. (See section outlined in Softwood Genetics/Molecular Biology-Gene Expression and Somatic Embryogenesis.

Degree of work is dependent on cooperation with researchers outside IPST and obtaining external funding. Expected to start by 94.4 and continue through 95.2)

This work will be carried out by the Forest Biology Technical Staff (Barbara Johns, Shannon Johnson, Yolanda Powell), Ph.D. student Renee Kapik, MS student Vincent Ciavatta , and Drs. Jerry Pullman and Ron Dinus. External funding will be sought for the gene expression research in cooperation with Dr. John Cairney and Dr. Jung Choi (Georgia Institute of Technology).

Softwood Genetics/Molecular Biology

Manipulation of Lignin Synthesis using Gene Technologies

We have isolated a cDNA clone of an enzyme (O-methyltransferase, OMT) which may be involved in lignin synthesis. By recloning this cDNA in inverse orientation ("antisense") and transferring it back into a plant, we hope to reduce or modify lignin synthesis. Two objectives pertain to this part of the project;

1. To construct vectors which contain the OMT cDNA in inverse orientation under the control of a constitutive promoter. (94.3)
2. To transfer these constructs into an easily regeneratable model plant such as tobacco. (94.4)

(These objective will be carried out in collaboration with colleagues at Texas A&M University)

Isolation of a genomic clone corresponding to the OMT cDNA will allow us to examine the signals which switch on the gene. Preliminary work from our lab suggests that there may be more than one copy of the gene, and excitingly one of these genes may be stem specific. If we could isolate a stem specific promoter then lignin-reducing antisense constructs could be expressed exclusively in the stem thus circumventing the potential problem of low-lignin plants being susceptible to pathogens and environmental stresses. A second part of the project will be;

3. To isolate genomic clones of the OMT. (Start by 94.4)
4. Isolate promoter fragments of the OMT gene. (Start by 95.1)

This work will be initiated by Dr. Shujun Chang, Assistant Scientist, and Dr. John Cairney. The project will be the subject of a grant proposal and its continuation will depend on acquiring external funding.

Drought-Stress Protection

Over expression of stress-related cDNA clones has been shown, in some cases, to confer enhanced stress tolerance upon herbaceous plants. This approach has not yet been tried in

conifers because of the difficulty of transformation and regeneration and because very few genes were available. We have recently cloned a number of stress-related genes and wish to attempt to express them in transgenic plants;

1. We will construct vectors suitable for expression of drought-related genes in transgenic plants (Yellow Poplar). (94.4)
2. We will transfer these constructs into Yellow Poplar, a model tree which is easily regenerated. (This work will be done in conjunction with Scott Merkle at UGA, Athens). (95.2)

This work will be carried out by Dr. John Cairney with assistance from Dr. Shujun Chang and Ms. Debbie Villalon. External funding has been sought to continue the project.

Regulation of Proteinase Inhibitor Gene expression

Proteinase Inhibitor (PI) genes are induced in plants undergoing insect attack, wounding and other environmental or chemical insults. The proteins encoded by these genes provide some level of protection to the plant and "overexpression" of proteinase inhibitor genes in tobacco renders that plant more resistant to attack by certain pathogens. I have isolated a PI gene from the shrub *Atriplex canescens*. This gene has an unusual pattern of expression in that it appears to be expressed through different classes of mRNA under different levels of stress. These different molecules may have different stabilities or translatabilities. We will determine the validity of these hypotheses by cloning the terminal region behind a control gene and determining whether expression of this gene is affected and by examining promoter control in the model plant *Arabidopsis thaliana*. Objectives:

1. Establish regeneration for Arabidopsis from tissue culture. (94.3)
2. Clone the coding region of the PI gene and express this in *E. coli.* (94.4)
3. Demonstrate that the PI gene product actually inhibits proteinase action in vivo. (94.4)
4. Generate terminal fragment by PCR and clone this into an expression vector. (94.4)
5. For promoter analysis, generate promoter fragment by PCR and clone this into an expression vector. (95.1)
6. Conduct transformation experiments using *Agrobacterium tumefaciens.* (95.1)

This work will be carried out by Ms. Debbie Villalon with assistance from Dr. John Cairney. External funding for this project will be sought.

Gene Expression and Somatic Embryogenesis

The technique of differential display allows minor difference in gene expression between sample tissues to be observed and representative clones to be isolated. We intend to apply these methods to somatic and zygotic embryogenesis

1. Somatic embryos of different stages will be isolated and RNA extracted for Reverse Transcription-PCR. (94.3)

2. RT-PCR will be carried out and reproducible differences in gene expression between different stages will be identified. ((95.1)
3. cDNA fragments identified above will be cloned and sequenced. We will try to identify these clones by comparison to the genebank. We hope that by identifying enzymes whose activities are elevated at different stages of embryogenesis we can modify media appropriately to increase the efficiency of somatic embryogenesis. (95.2)

This work will be carried out by Dr. John Cairney in collaboration with Dr. Gerald Pullman. This project is large in scale and implication. Its continuation will depend on the ability to acquire external funding.

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - INITIATION

Gerald Pullman
Shannon Johnson
Barbara Johns
Yolanda Powell

March 21-22, 1995

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - INITIATION

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During the Spring 1994 PAC Review it was reported that our goal of approximately 35-40% average extrusion had been approached, but that only 1% of the explants actually initiated. In many explants the start of the initiation process was clearly visible, but new somatic embryos did not continue to grow and multiply. It was proposed that the focus during 1994 would be to understand what factors are inhibiting the continued growth of the first somatic embryos formed during initiation.

As part of initiation experimentation during the early part of 1994, somatic embryos were included in our tests in order to develop a somatic initiation model. The hypothesis was that one could use well-developed somatic embryos from liquid culture (stages 2-4) to evaluate initiation and growth potentials of new initiation media. Single somatic embryos at stages 2-4 were selected and placed on initiation medium. Somatic embryos could be tested year round, were expected to be genetically identical, and required less labor for preparation. Initial experiments showed little to no somatic growth to form a colony even though new somatic embryos were observed starting to grow on the surface of the somatic embryo explant. When control media were run without activated charcoal it was observed that some explants formed new somatic embryos and these continued to grow to form a colony. This observation suggested the hypothesis that activated charcoal adsorbs a required media component. If a required media component is adsorbed by the activated charcoal over time one might expect to observe growth starting but then stopping as the explant reserve of required nutrient is used up and as the activated charcoal continues to adsorb the necessary nutrient.

With the above hypothesis in mind a three point approach was developed to identify the possible missing component(s).

1) Analyze initiation medium for major and minor elements.
2) Prepare the base medium and add single elements (double the normal concentration) to compensate for possible adsorption. Test initiation with the somatic initiation model. Also test media with all micronutrients doubled or tripled in case multiple elements are missing.
3) Test initiation with zygotic embryos for the same media prepared in approach #2.

ANALYSIS Treatments used for the analysis of metals in initiation media were based on two ideas: 1) charcoal, as stated above, may adsorb necessary nutrients and 2) gelrite, a highly purified heteropolysaccharide, forms clear gels with the aid of soluble salts. Control media (in the above experiment) without activated charcoal contained 2.5 g/l gelrite while charcoal media contained 4 g/l. For the metal analysis, four treatments were prepared based on the charcoal containing medium (201) that has produced 35-40% extrusion in zygotic embryo experiments.

Two variables were tested in a 2 x 2 factorial arrangement: 1) charcoal concentration - 0 or 2.5 g/l and 2) gelrite concentration - 2 or 4 g/l (Medium 201, 278, 279 and 280 - Table 1).

Media were poured and then allowed to "age" for ten days. It has been observed that around day ten the zygotic embryo begins to extrude from the ovule and comes in direct contact with the initiation medium. To prepare samples for the analysis, gelled media were pulled through a filter and the liquid collected. Each treatment was replicated three times. A water control was also included for a total of 13 samples. Samples were analyzed for total recoverable metals using inductively coupled plasma (ICP) - atomic emission spectroscopy (for K, Ca, Na, Mg, Mn, Fe, Zn, and Ni) and graphite furnace atomic adsorption (for B, Co and Cu).

Results are shown in Figures 1, 2 and 3 and Tables 2 and 3. To summarize, gelrite adds low levels of K, Na and Fe to initiation media while decreasing the levels of Mg, Mn and Zn. The addition of activated charcoal to the media increases the amount of K and Ni and decreases the amount of Ca, Zn and Cu. The most dramatic results show the reduction of copper by 90% and zinc by 50% in charcoal containing medium. These observations became the spring board for subsequent initiation experiment hypotheses.

SOMATIC INITIATION Somatic embryos were utilized in initiation experiments prior to the arrival of 1994 summer cone collections. The first somatic experiment involved the addition of two times the micronutrients (medium 274 - Table 1) to basal medium 201. Results showed a statistically significant increase in colony size of double micronutrients over basal amounts (Figure 4). The next experiment concentrated on tripling the micronutrient concentration or the addition of single microelements to double micronutrient medium 274. Basal 201 and a medium without charcoal were used as controls. Results are shown in Figure 5. Medium 274 again had larger colony sizes over 201. Tripling the micronutrients and the single addition of microelements did not increase this size further. The medium without charcoal produced significantly larger colony sizes than other treatments.

At this point in the program we received the information from the above metal analysis experiment. It provided the hypothesis that even higher copper and zinc levels than 2X might be needed in the initiation media. Six levels of copper (0.125, 0.25, 0.375, 0.5, 1.0 and 2.5 mg/l) were used in the next somatic initiation experiment. Colony size increased with increasing copper concentration (Figure 6). This result was very encouraging but it was still not clear what the final (free) copper concentration was and if levels needed to go even higher.

FOLLOW-UP ANALYSIS Another metal analysis was performed using 6 media that increased both the initial copper and zinc levels (Table 4). Liquid was collected from the gelled media on days 14 and 28 to investigate whether or not copper and zinc continued to be adsorbed by the charcoal over time. Each treatment was replicated three times and a water control was included for a total of 37 samples. Results from the first analysis of zinc and copper (day 10 - medium 201) are included along with the current results for comparison (Figures 7 and 8).

In general, free copper and zinc levels rise when the initial concentrations are increased. The level of free copper available in medium without charcoal was duplicated by adding 2.5 mg/l of $CuSO_4$ $5H_2O$ to charcoal containing medium. When the initial zinc level was quadrupled, it more than compensated for the adsorption by charcoal. Doubling the $ZnSO_4$ $7H_2O$ to 28.8 mg/l would probably result in the desired level. There was a statistically significant difference between the amount of free zinc available on days 14 and 28 with the latter having higher concentrations. No such difference was found with the copper.

Components	Media (mg/l)				
	201	278	279	280	274
NH_4NO_3	200.0	200.0	200.0	200.0	200.0
KNO_3	909.9	909.9	909.9	909.9	909.9
KH_2PO_4	136.1	136.1	136.1	136.1	136.1
$Ca(NO_3)_2 \bullet 4H_2O$	236.2	236.2	236.2	236.2	236.2
$MgSO_4 \bullet 7H_2O$	246.5	246.5	246.5	246.5	246.5
$Mg(NO_3)_2 \bullet 6H_2O$	256.5	256.5	256.5	256.5	256.5
$MgCl_2 \bullet 6H_2O$	101.7	101.7	101.7	101.7	101.7
KI	4.15	4.15	4.15	4.15	8.3
H_3BO_3	15.5	15.5	15.5	15.5	31
$MnSO_4 \bullet H_2O$	10.5	10.5	10.5	10.5	21
$ZnSO_4 \bullet 7H_2O$	14.4	14.4	14.4	14.4	28.8
$Na_2MoO_4 \bullet 2H_2O$	0.125	0.125	0.125	0.125	0.25
$CuSO_4 \bullet 5H_2O$	0.125	0.125	0.125	0.125	0.25
$CoCl_2 \bullet 6H_2O$	0.125	0.125	0.125	0.125	0.25
$FeSO_4 \bullet 7H_2O$	13.9	13.9	13.9	13.9	13.9
Na_2EDTA	18.65	18.65	18.65	18.65	18.65
Maltose	15,000	15,000	15,000	15,000	15,000
myo-Inositol	20,000	20,000	20,000	20,000	20,000
Casamino acids	500	500	500	500	500
L-Glutamine	450	450	450	450	450
Thiamine•HCl	1.0	1.0	1.0	1.0	1.0
Pyridoxine•HCl	0.5	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5	0.5
Glycine	2.0	2.0	2.0	2.0	2.0
2,4-D	220	220	1.1	1.1	220
BAP	90	90	0.45	0.45	90
Kinetin	86	86	0.43	0.43	86
Activated charcoal	2500	2500	--	--	2500
Gelrite	4000	2000	4000	2000	4000
pH	5.2	5.2	5.2	5.2	5.2

Table 1. Composition of media 201, 278, 279 and 280 (treatments used in metal analysis experiment) and medium 274 (double the micronutrients of medium 201) used in a somatic initiation experiment.

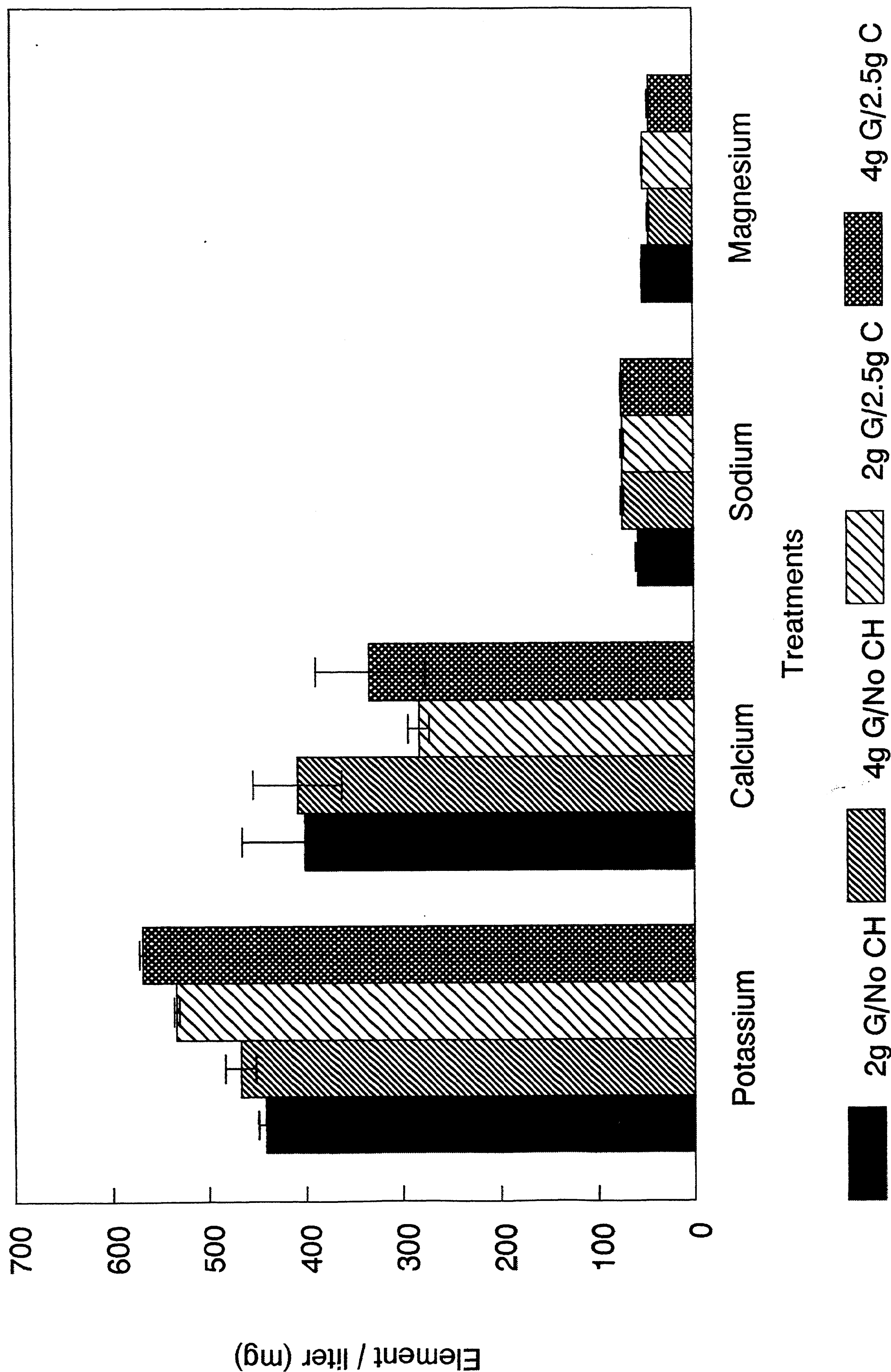

Figure 1. Effect of two gelrite and two charcoal concentrations on the amount (mg/l) of macronutrients (K, Ca, Na and Mg) available in initiation media.

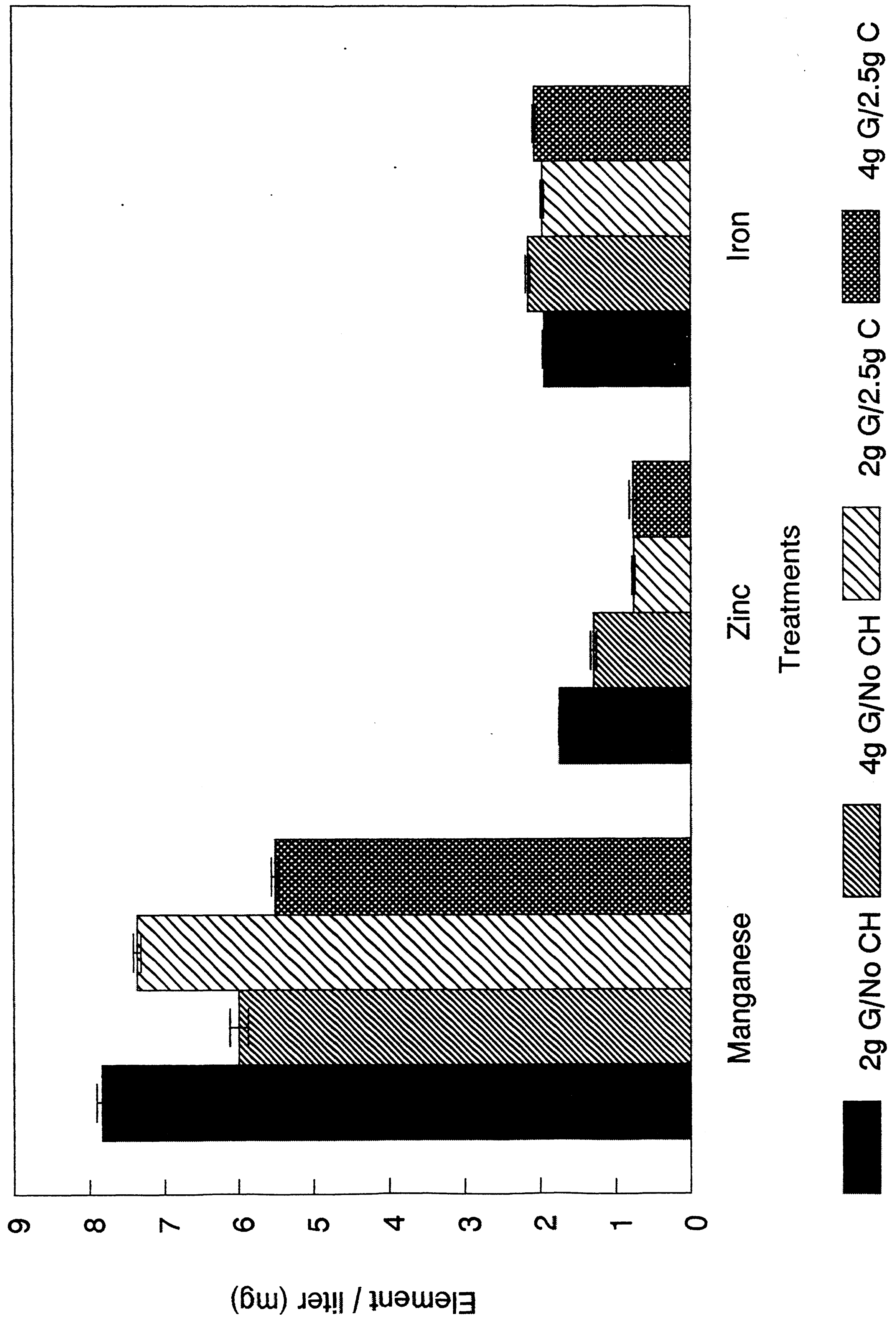

Figure 2. Effect of two gelrite and two charcoal concentrations on the amount (mg/l) of micronutrients (Mn, Zn and Fe) available in initiation media. Note the 50% reduction in the amount of Zn when charcoal is added.

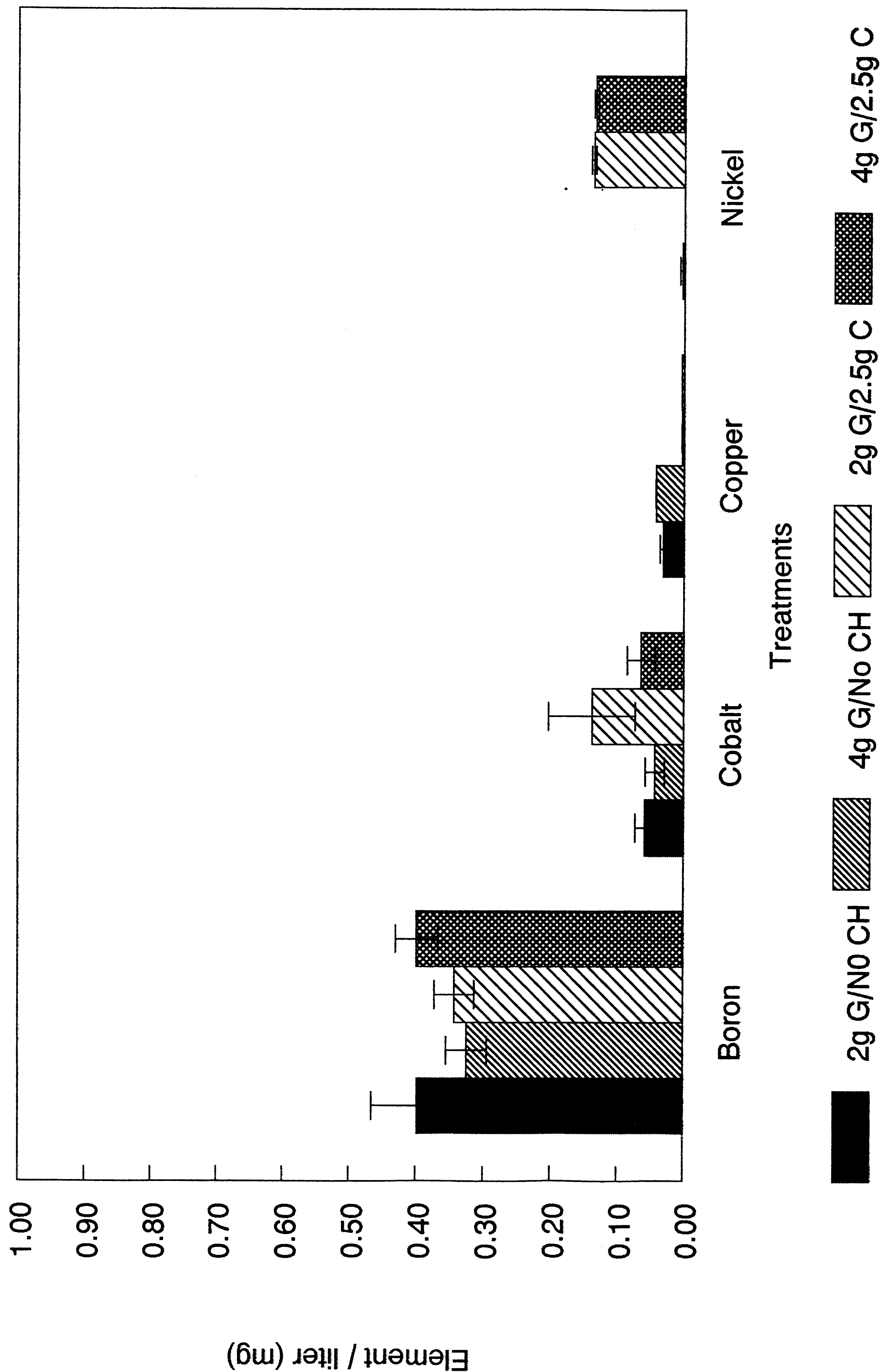

Figure 3. Effect of two gelrite and two charcoal concentrations on the amount (mg/l) of micronutrients (B, Co, Cu and Ni) available in initiation media. Note the 90% reduction in the amount of copper when charcoal is added.

Element	Gelrite 2g/L	4g/L	% Change	Significant at 95%
	mg/L:	mg/L:		
K	487.36	517.45	6.17	yes
Ca	341.36	369.83	8.34	no
Na	59.18	73.72	24.57	yes
Mg	52.75	46.07	-12.66	yes
Mn	7.59	5.75	-24.24	yes
Fe	1.95	2.12	8.72	yes
Zn	1.26	1.04	-17.46	yes
	µg/L:	µg/L:		
B	370.33	370.33	0.00	no
Co	98.53	54.07	-45.12	no
Cu	17.33	23.17	33.70	no
Ni	70	66.67	-4.76	no

Table 2. Percent change in the amount of available macro and micronutrients between the two gelrite concentrations. Amount of each element is an average of the three replications. An analysis of variance was performed to determine significance at 95%.

Element	Charcoal 0 g/L	2.5 g/L	% Change	Significant at 95%
	mg/L:	mg/L:		
K	454.16	550.65	21.25	yes
Ca	403.66	307.53	-23.81	yes
Na	65.67	67.23	2.38	no
Mg	49.52	49.3	-0.44	no
Mn	6.91	6.43	-6.95	yes
Fe	2.05	2.02	-1.46	no
Zn	1.53	0.78	-49.02	yes
	µg/L:	µg/L:		
B	361.33	379.33	4.98	no
Co	51.43	101.17	96.71	no
Cu	36.88	3.62	-90.18	yes
Ni	1.67	135	7983.83	yes

Table 3. Percent change in the amount of available macro and micronutrients between the two charcoal concentrations. Amount of each element is an average of the three replications. An analysis of variance was performed to determine significance at 95%. Note the reduction of zinc and copper when charcoal is added.

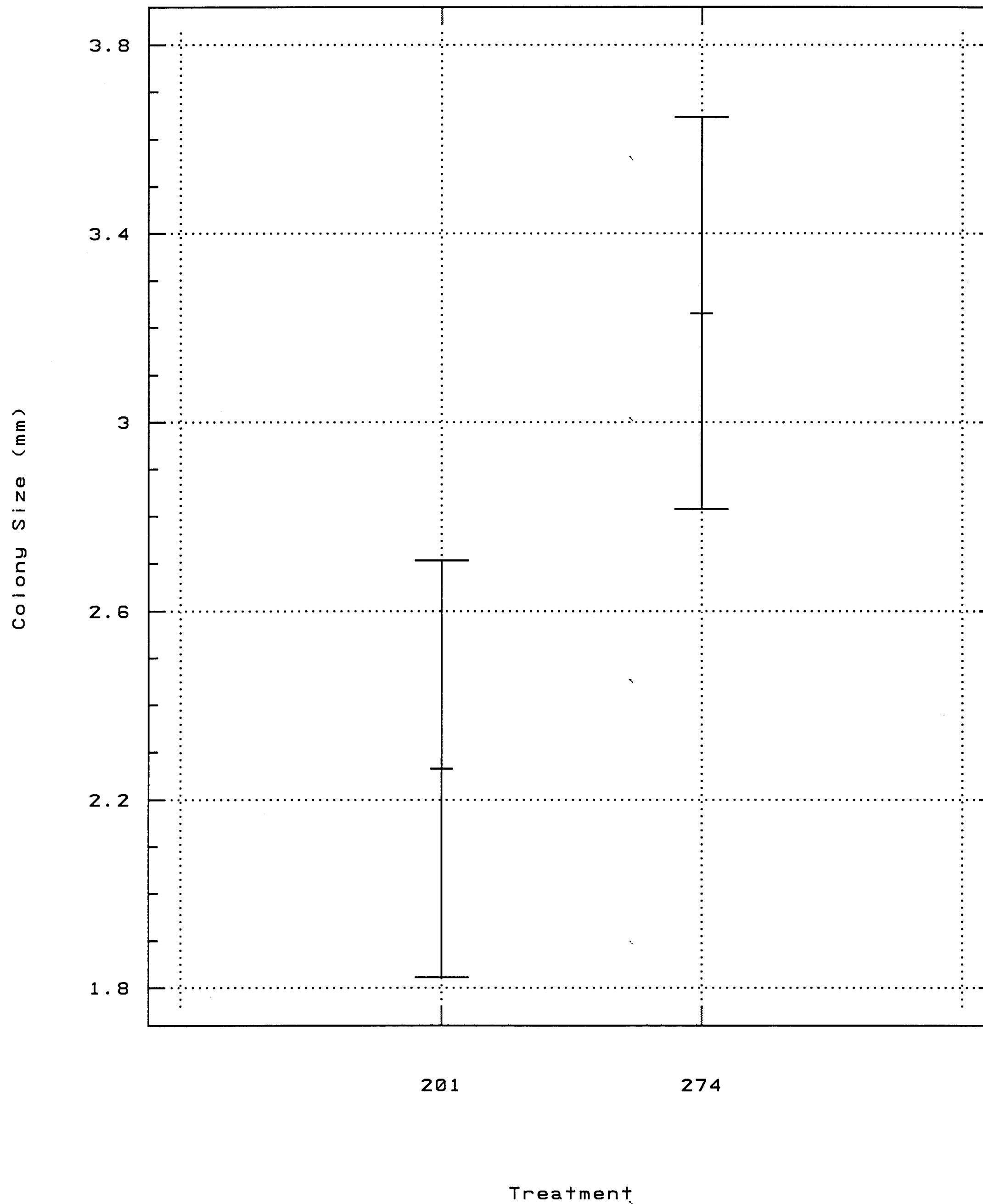

Figure 4. Confidence intervals for basal medium 201 and medium 274 (201 with double the micronutrients). A statistically significant increase in colony size is shown with double the micronutrients treatment.

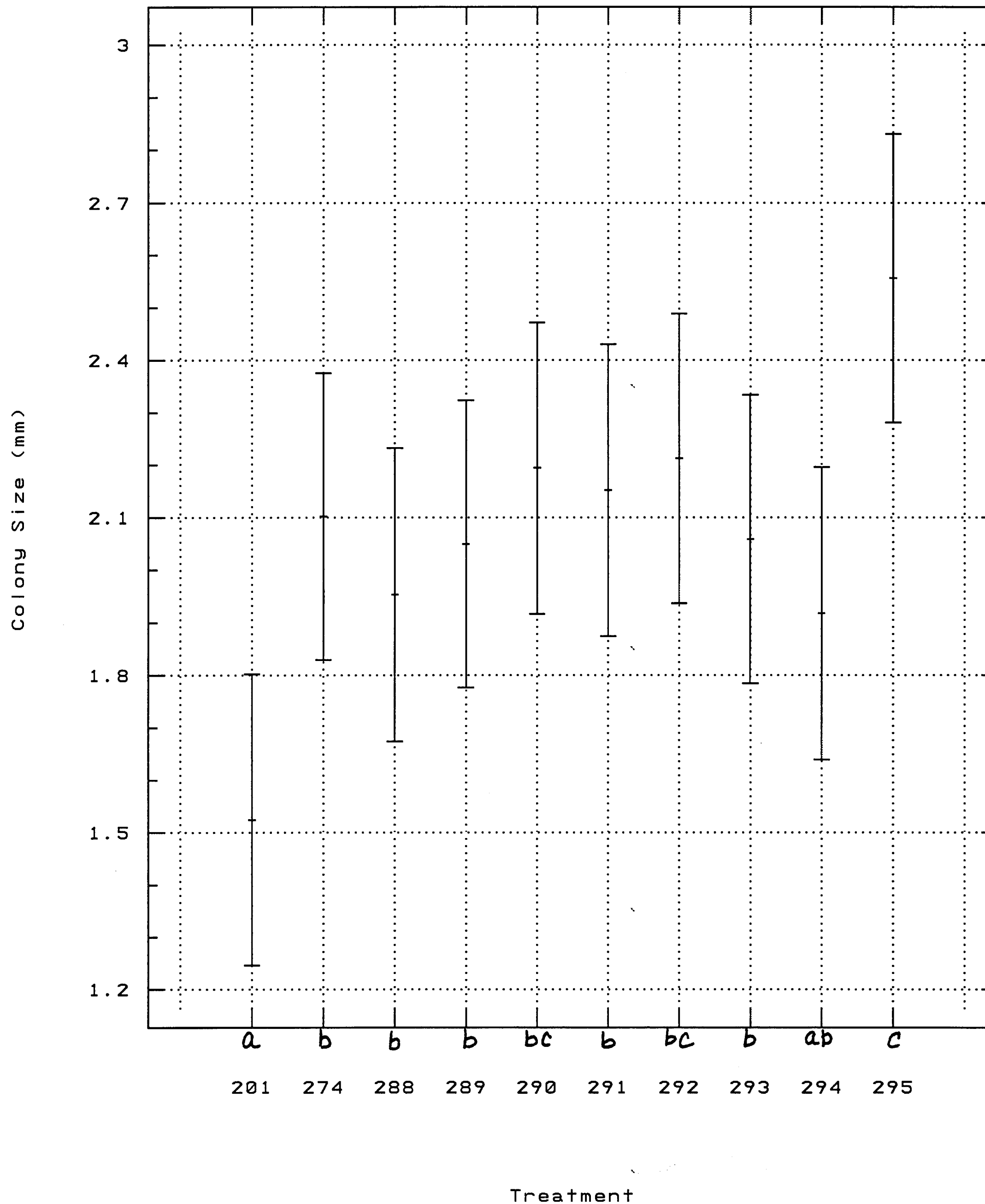

Figure 5. Confidence interval for basal medium 201, double micronutrients medium 274 and a control, without charcoal medium 295. Media 288 - 294 represent treatments with 3X micronutrients or the addition of a single micronutrient. Treatments with the same letter are not significantly different (Duncan's multiple range test, P = 0.05).

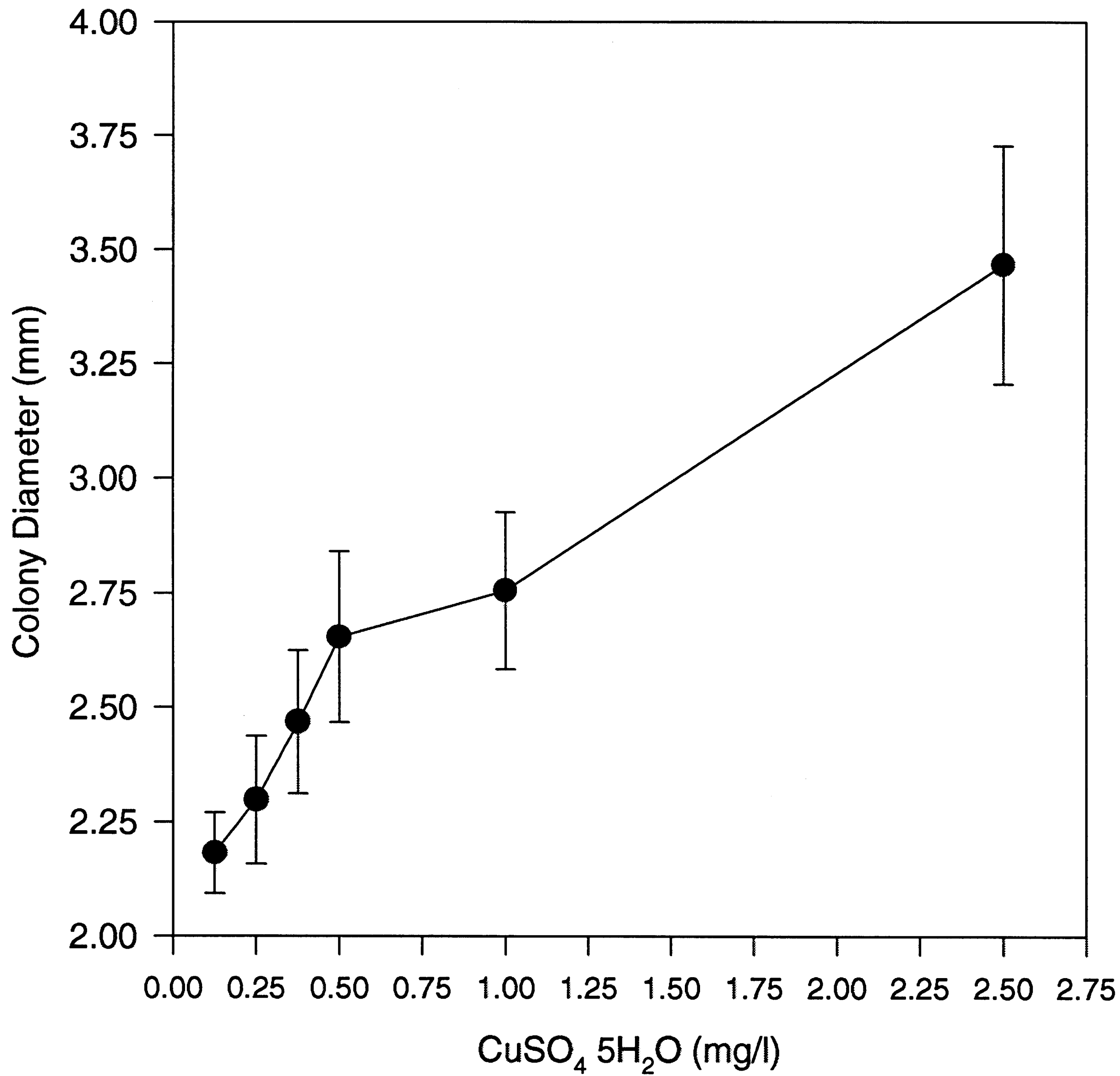

Figure 6. Effect of initial $CuSO_4$ $5H_2O$ concentration in initiation media on the colony diameter of somatic embryos.

Components	Media (mg/l)					
	375	372	354	355	373	374
NH_4NO_3	200.0	200.0	200.0	200.0	200.0	200.0
KNO_3	909.9	909.9	909.9	909.9	909.9	909.9
KH_2PO_4	136.1	136.1	136.1	136.1	136.1	136.1
$Ca(NO_3)_2 \bullet 4H_2O$	236.2	236.2	236.2	236.2	236.2	236.2
$MgSO_4 \bullet 7H_2O$	246.5	246.5	246.5	246.5	246.5	246.5
$Mg(NO_3)_2 \bullet 6H_2O$	256.5	256.5	256.5	256.5	256.5	256.5
$MgCl_2 \bullet 6H_2O$	101.7	101.7	101.7	101.7	101.7	101.7
KI	4.15	4.15	4.15	4.15	4.15	4.15
H_3BO_3	15.5	15.5	15.5	15.5	15.5	15.5
$MnSO_4 \bullet H_2O$	10.5	10.5	10.5	10.5	10.5	10.5
$ZnSO_4 \bullet 7H_2O$	14.4	57.6	57.6	57.6	57.6	57.6
$Na_2MoO_4 \bullet 2H_2O$	0.125	0.125	0.125	0.125	0.125	0.125
$CuSO_4 \bullet 5H_2O$	0.125	0.25	1.0	2.5	5.0	10.0
$CoCl_2 \bullet 6H_2O$	0.125	0.125	0.125	0.125	0.125	0.125
$NiCl_2 \bullet 6H_2O$	0.13	0.13	0.13	0.13	0.13	0.13
$FeSO_4 \bullet 7H_2O$	13.9	13.9	13.9	13.9	13.9	13.9
Na_2EDTA	18.65	18.65	18.65	18.65	18.65	18.65
Maltose	15,000	15,000	15,000	15,000	15,000	15,000
myo-Inositol	20,000	20,000	20,000	20,000	20,000	20,000
Casamino acids	500	500	500	500	500	500
L-Glutamine	450	450	450	450	450	450
Thiamine•HCl	1.0	1.0	1.0	1.0	1.0	1.0
Pyridoxine•HCl	0.5	0.5	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5	0.5	0.5
Glycine	2.0	2.0	2.0	2.0	2.0	2.0
2,4-D	1.1	220	220	220	220	220
BAP	0.45	90	90	90	90	90
Kinetin	0.43	86	86	86	86	86
Activated charcoal	--	2500	2500	2500	2500	2500
Gelrite	4000	4000	4000	4000	4000	4000
pH	5.7	5.2	5.2	5.2	5.2	5.2

Table 4. Composition of the six treatments used in the follow-up ICP analysis of zinc and copper.

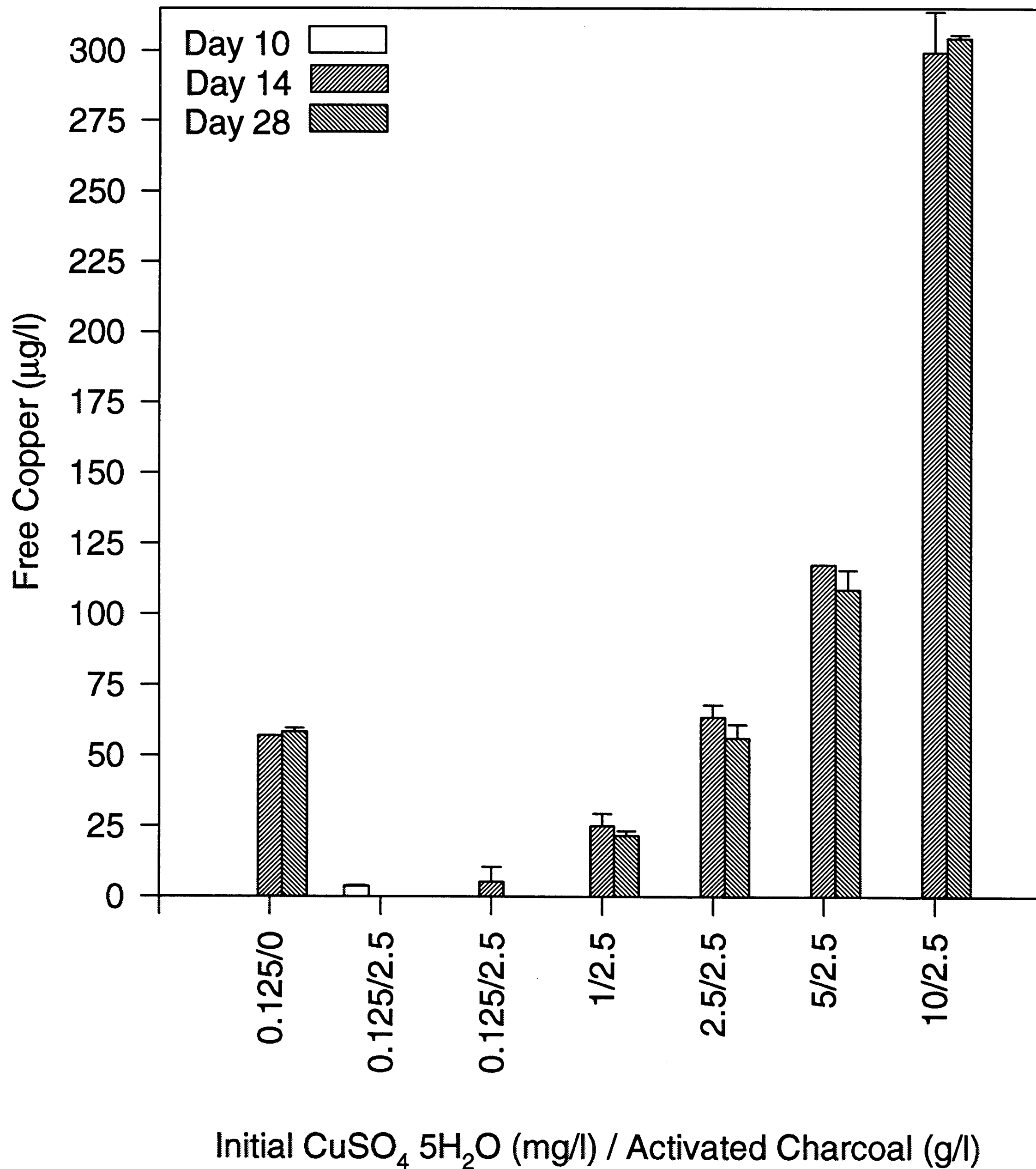

Figure 7. Effect of five $CuSO_4$ $5H_2O$ and two charcoal concentrations on the amount (μg/l) of free Cu available in initiation media.

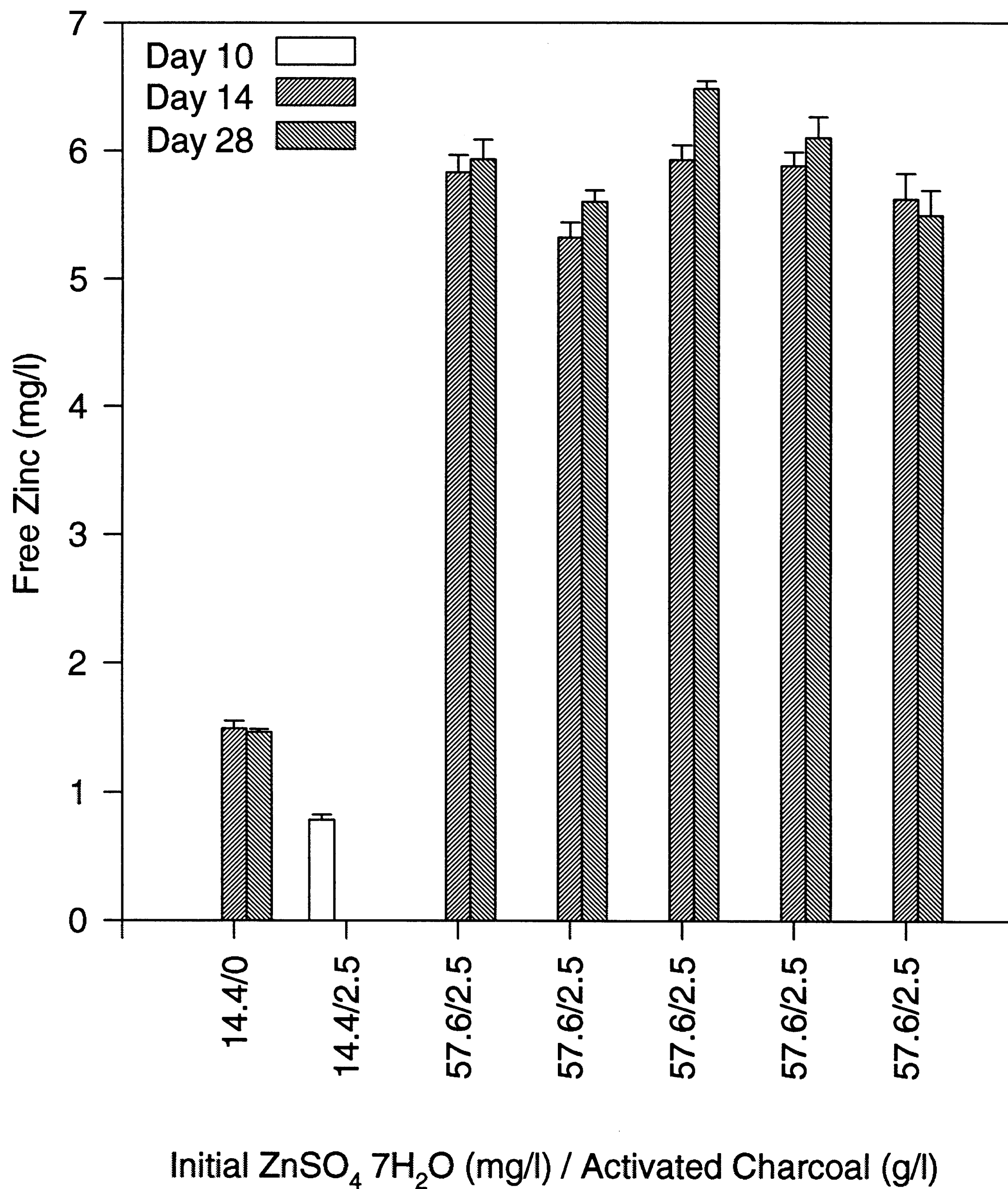

Figure 8. Effect of two $ZnSO_4$ $7H_2O$ and two charcoal concentrations on the amount (mg/l) of free Zn available in initiation media.

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - INITIATION: March 1995 Update

Gerald Pullman
Barbara Johns
Shannon Johnson
Yolanda Powell
Steve Van Winkle

March 21-22, 1995

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - INITIATION: MARCH 1995 UPDATE

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LOBLOLLY PINE - ZYGOTIC INITIATION Summer 1994 zygotic initiation experiments were evaluated after the 1994 Fall PAC during the months of November and December. Zygotic embryos were used to test some of the same treatments involved in the prior somatic embryo experiments. Unfortunately, adding the extra copper and zinc to our charcoal media did not translate into increased initiations of the zygotic embryos. From our previous experiments we know that extra copper and zinc are important to somatic embryo growth, but there must be more unknown roadblocks preventing us from getting the higher initiation rates desired. Summer 1995 initiation experiments will give us another chance to tackle those obstacles.

NORWAY SPRUCE - ZYGOTIC INITIATION In an earlier attempt to develop a model initiation system, a charcoal medium (59, see table 1) was used with Norway Spruce. This medium was tested against a clear initiation medium (56, see table 1). The results of this experiment are summarized below:

Media #	# of Initiations / Total	% Initiation
56	8 / 50	16
59	3 / 50	6

Although the results are not statistically significant, they imply that charcoal is somehow impeding Norway Spruce initiation.

Armed with the knowledge that copper and zinc in a charcoal medium were found to be at deficient levels for loblolly pine, an "improved" medium 59 was tested at the end of 1994. Copper was added at twenty times the normal amount while zinc was doubled. This became charcoal medium 398 (see table 1). The results of this experiment are summarized below:

Media #	# of Initiations / Total	% Initiation
56	8 / 30	27
59	5 / 29	17
398	8 / 29	28

As in the first trial, the clear medium 56 appears to produce more initiations than the charcoal medium 59. When the extra copper and zinc are added to medium 398, the level of initiation appears to increase back up to the standard of the clear medium.

Components	Media (mg/l)		
	56	**59**	**398**
KCl	372.5	372.5	372.5
KNO_3	50	50	50
KH_2PO_4	85	85	85
$MgSO_4 \bullet 7H_2O$	160	160	160
$CaCl_2 \bullet 6H_2O$	220	220	220
KI	0.145	0.145	0.145
H_3BO_3	3.1	3.1	3.1
$MnSO_4 \bullet H_2O$	8.45	8.45	8.45
$ZnSO_4 \bullet 7H_2O$	4.3	4.3	**8.6**
$Na_2MoO_4 \bullet 2H_2O$	0.125	0.125	0.125
$CuSO_4 \bullet 5H_2O$	0.0125	0.0125	**0.25**
$CoCl_2 \bullet 6H_2O$	0.0125	0.0125	0.0125
$FeSO_4 \bullet 7H_2O$	13.9	13.9	13.9
Na_2EDTA	18.65	18.65	18.65
Sucrose	10,000	10,000	10,000
myo-Inositol	50	50	50
Casamino acids	500	500	500
L-Glutamine	750	750	750
Thiamine•HCl	0.05	0.05	0.05
Pyridoxine•HCl	0.05	0.05	0.05
Nicotinic acid	0.25	0.25	0.25
L-Asparagine	50	50	50
NAA	2.0	**100**	**100**
BAP	1.0	**50**	**50**
Activated Charcaol	--	**1,250**	**1,250**
Difco Agar	7,000	7,000	7,000
pH	5.8	5.8	5.8

Table 1. Composition of the six treatments used in Norway Spruce initiation experiments.

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

Growth Kinetics of Loblolly Pine Early-Stage Embryo Suspensions

Gerald Pullman
Barbara Johns
Shannon Johnson
Yolanda Powell

March 21-22, 1995

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

GROWTH KINETICS OF LOBLOLLY PINE EARLY-STAGE EMBRYO SUSPENSIONS

Gerald Pullman
Barbara Johns
Shannon Johnson
Yolanda Powell

INTRODUCTION

To better understand the physical environment of liquid Loblolly pine cell suspension cultures several embryo parameters were monitored: medium pH, osmolality, electrical conductivity, settled cell volume, cell fresh and dry weight, and embryo stage. To date three genotypes (71, 41, and 195) have been monitored over a single extended culture cycle.

METHOD AND MATERIALS

Three genotypes maintained on medium 16 (Table 1) were subcultured to four replicates of 1.0 liter bottles each at a 1:9 density in fresh medium 16 and held on a shaker in the dark. At the sample date (within 1 hour of addition of cells, 1, 2, 3, 5, 7, 9, 12, 15, and 19 days) the bottle was swirled and 25 mls of culture was removed. One ml was immediately pipeted from the sample for observation of embryo appearance and stage rating.

The cells were settled for 20 minutes and the volume recorded. Ten mls of medium was used to determine pH, osmolality, and electrical conductivity. The remaining cells in medium were poured onto a preweighed GLA-5000 filter placed in a Buchner funnel over a low vacuum. The cells were rinsed once with distilled water. The filter and cells were then placed in a labeled preweighed glass petri plate and fresh weight was recorded. The cells were dried in a 70° C oven overnight. The plates were put in a desicator with desicant (to inhibit water vapor uptake), allowed to cool and weighed quickly to obtain dry weight of cells.

Genotype 71 was evaluated first beginning on 1/26/94 and genotypes 195 and 41 on 2/23/94.

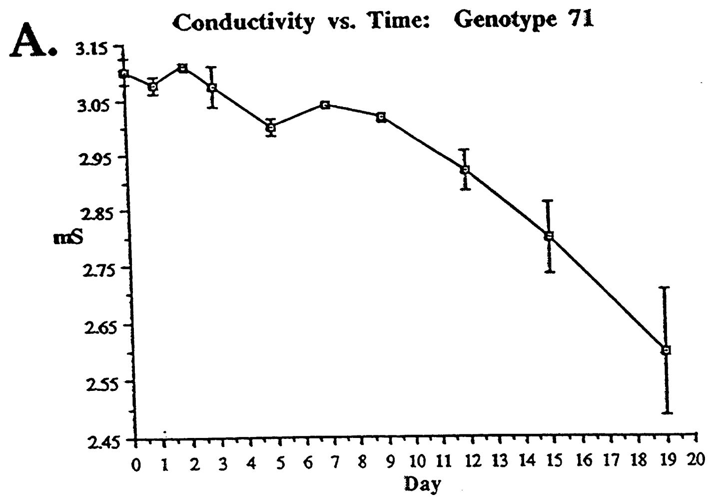
RESULTS AND DISCUSSION

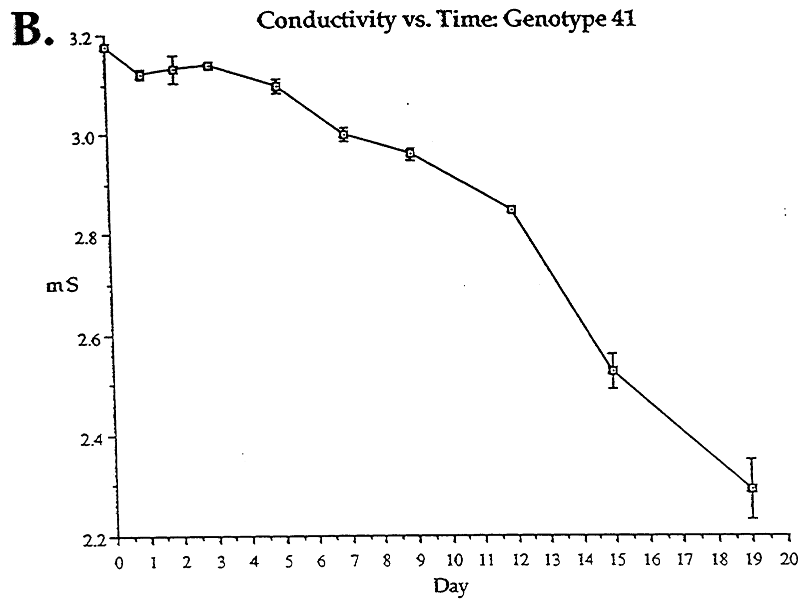
Data for the three genotypes is consistent and within each genotype the standard error bars are fairly small. The electrical conductivity values for the cell suspensions began approximately at 3.15 mS and gradually declined to 2.5 mS indicating that ionic components of the media were being steadily used. The osmolality showed a striking pattern of steadily rising mmol/kg from approximately 155 to about 210 mmol/kg peak at 9-10 days decreasing to 160-180 mmol/kg. The cleavage of sucrose to fructose and glucose probably accounts for a major part of the rise. Settled cell volumes, and fresh and dry weights of the cell suspensions showed a 3-4 day lag followed by a linear increase. Embryo stage increased from day 5 onward but was more variable among the genotypes. Over the first five days the pH showed a steep decline from 5.4 to 4.5 but by day 19 had slowly increased back to 5.4.

All embryo parameters show a steady continuation of growth after a short lag. However there appears to be a major shift in media components at day 9-10 when osmolality reaches a peak and with the pH change at day 5. The breakdown of sucrose and the cell use of $NH4^+$ releases H^+ ions. This probably occurs in the first few days until the $NH4^+$ uptake is inhibited or becomes unavailable to the cells. As NO3 is used pH rises. It does not appear to be detrimental to grow cell suspensions over an extended culture cycle. As osmolality and pH requirements of developing embryos becomes known this data will help the Tissue Culture program construct protocols which manipulate osmolality (sucrose vs. maltose or glucose) and pH ($NH4^+$ vs. $NO3^-$).

	16
NH_4NO_3	603.8
KNO_3	909.9
$Ca(NO_3)_2 \bullet 4H_2O$	236.2
KH_2PO_4	136.1
$MgSO_4 \bullet 7H_2O$	246.5
$Mg(NO_3)_2 \bullet 6H_2O$	256.5
$MgCl_2 \bullet 6H_2O$	101.7
$MnSO_4 \bullet H_2O$	10.5
$ZnSO_4 \bullet 7H_2O$	14.4
$CuSO_4 \bullet 5H_2O$	0.125
$FeSO_4 \bullet 7H_2O$	6.95
Na_2EDTA	9.33
H_3BO_3	15.5
$NaMoO_4 \bullet 2H_2O$	0.125
$CoCl_2 \bullet 6H_2O$	0.125
KI	4.15
myo-Inositol	1.0g
Thiamine•HCl	1.0
Nicotinic acid	0.5
Pyridoxine•HCl	0.5
Glycine	2.0
L-Glutamine	450.0
Casamino acids	500.0
Sucrose	30.0g
2,4-D	1.1
BAP	0.45
Kinetin	0.43
pH	5.7

TABLE 1. Medium composition for 16 used in growth kinetics liquid cell suspension experiments.





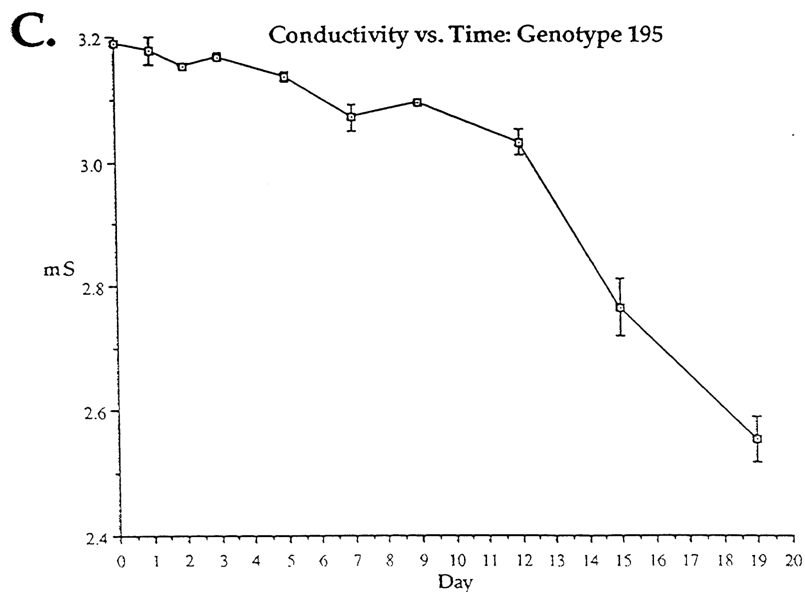
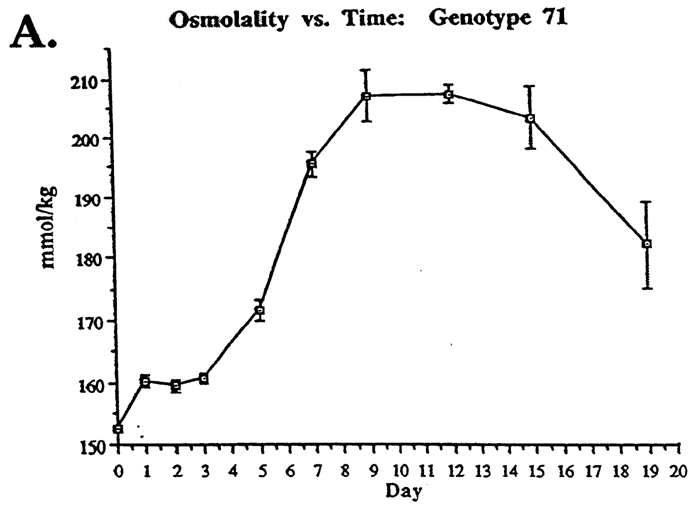
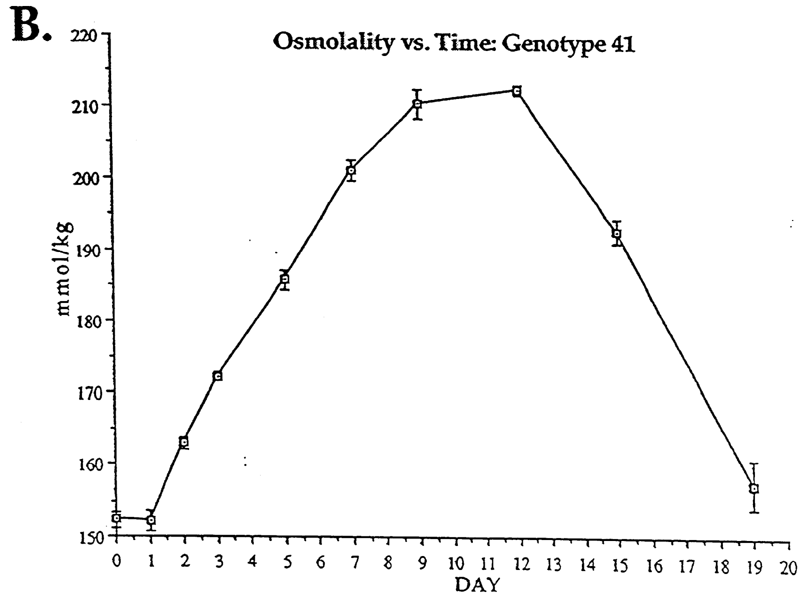


Figure 1. Medium electrical conductivity for three genotypes of Loblolly pine suspension cultures over a 19 day subculture. **A.** Genotype 71 **B.** Genotype 41 **C.** Genotype 195.





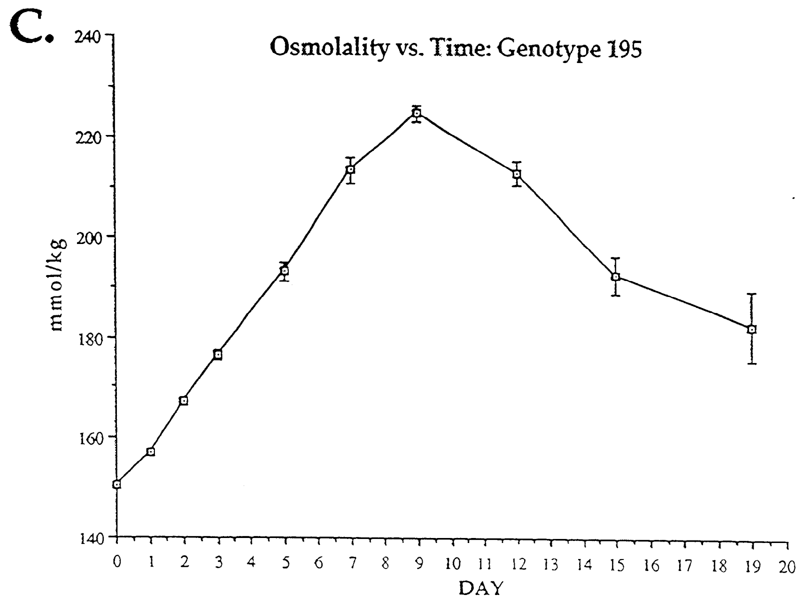
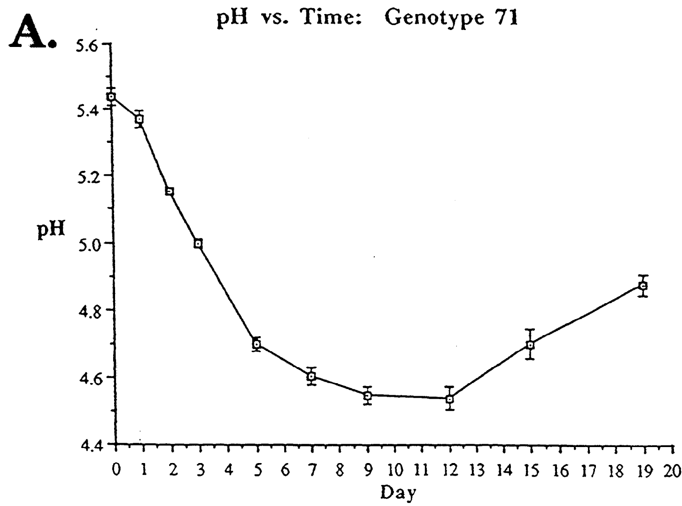
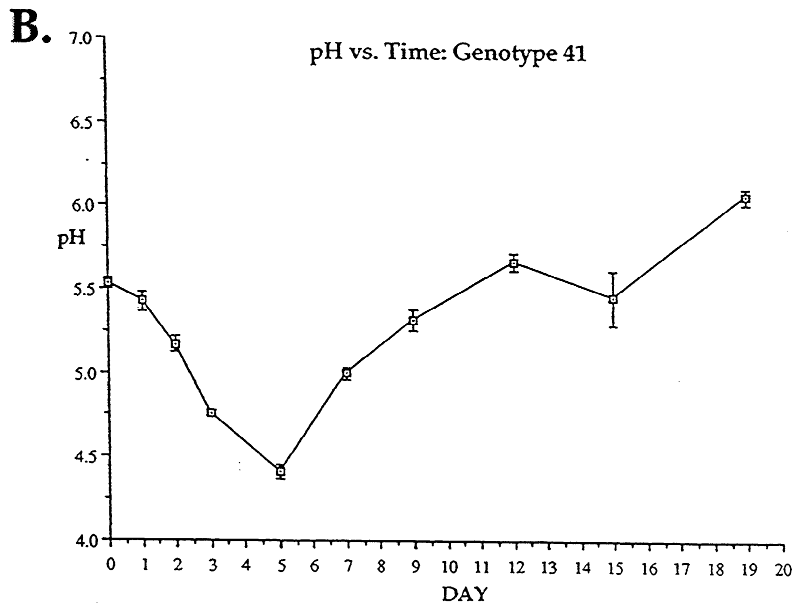


Figure 2. Medium osmolality for three genotypes of Loblolly pine suspension cultures over a 19 day subculture. **A.** Genotype 71 **B.** Genotype 41 **C.** Genotype 195.





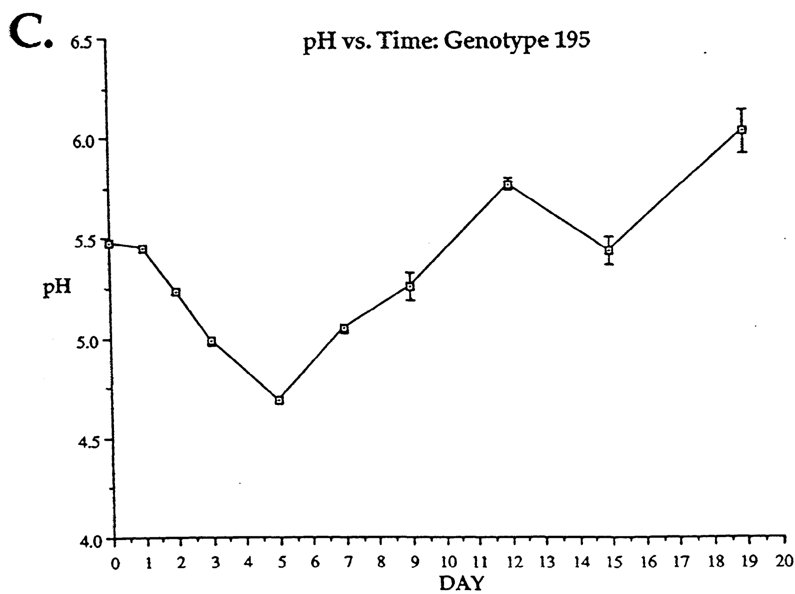
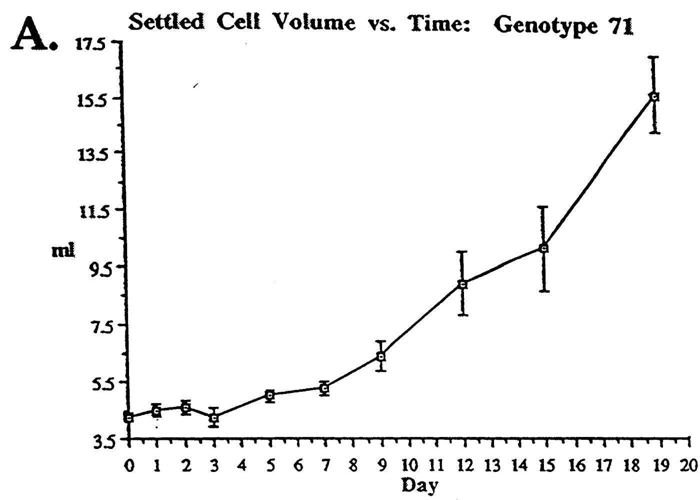
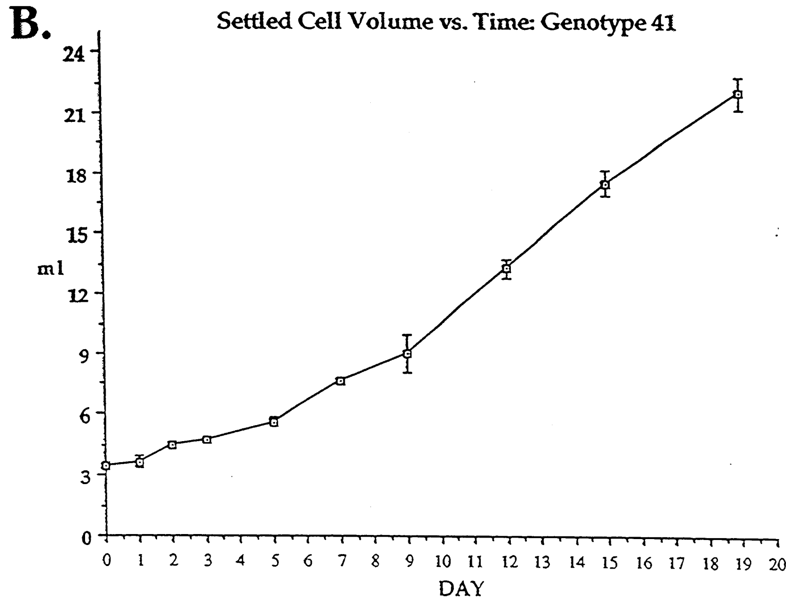


Figure 3. Medium pH for three genotypes of Loblolly pine suspension cultures over a 19 day subculture. **A.** Genotype 71 **B.** Genotype 41 **C.** Genotype 195.





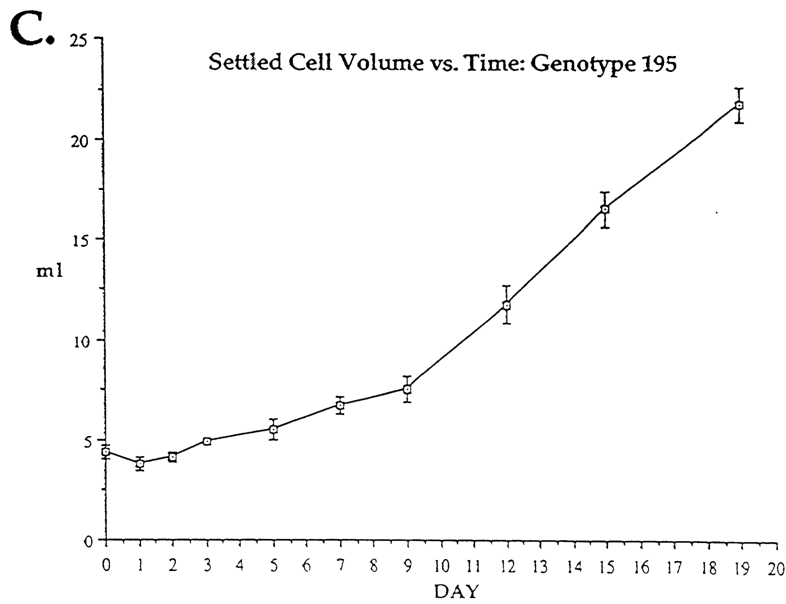
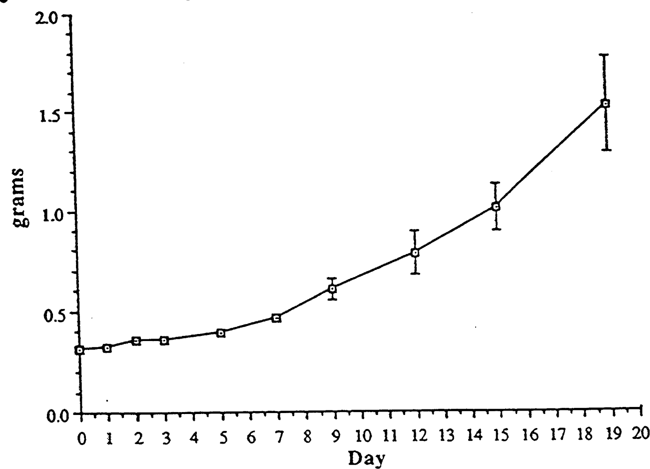
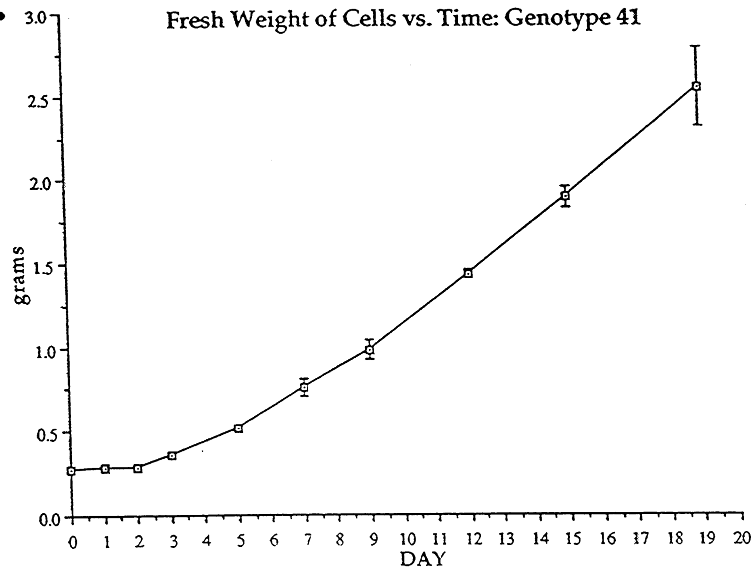


Figure 4. Settled cell volume for three genotypes of Loblolly pine suspension cultures over a 19 day subculture. **A.** Genotype 71 **B.** Genotype 41 **C.** Genotype 195.





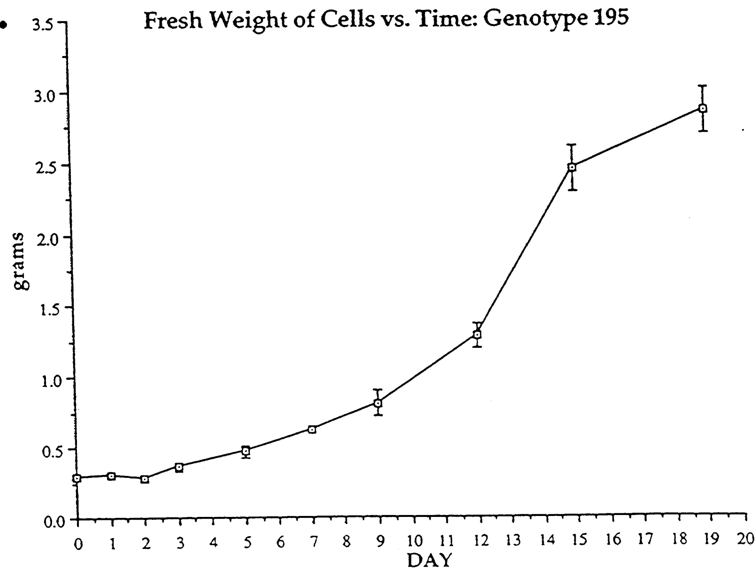
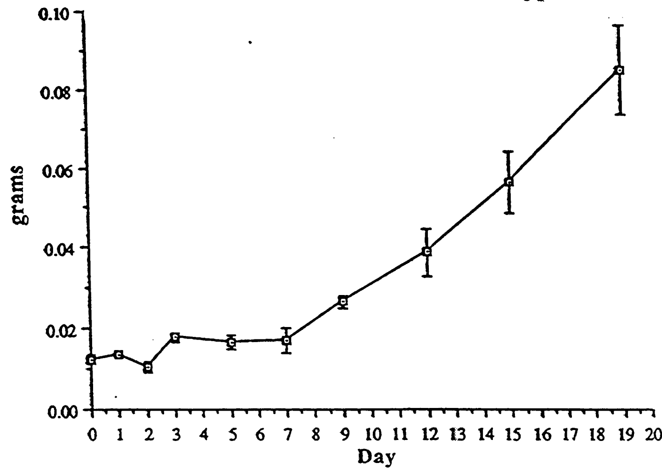
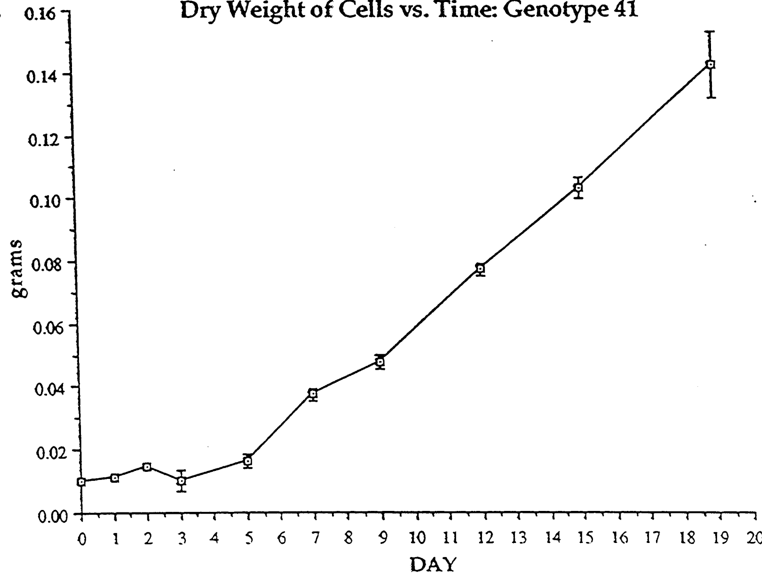


Figure 5. Fresh weight of cells for three genotypes of Loblolly pine suspension cultures over a 19 day subculture. **A.** Genotype 71 **B.** Genotype 41 **C.** Genotype 195.





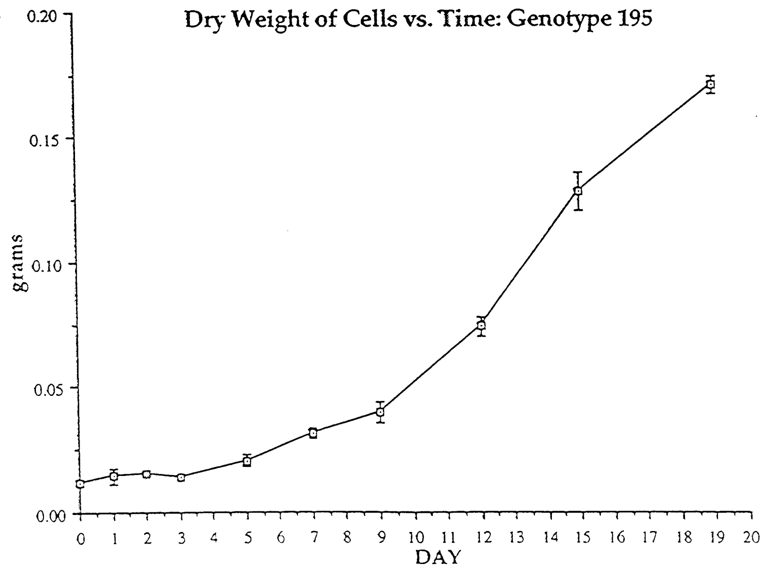
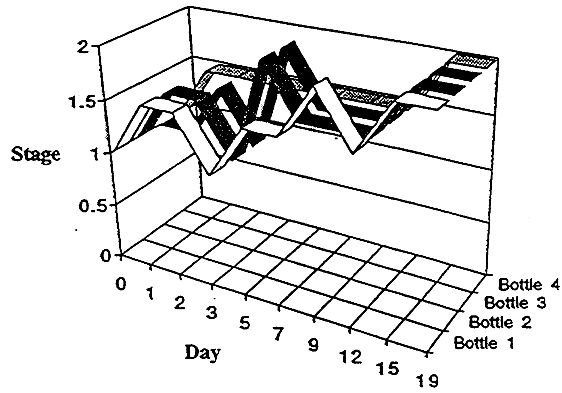
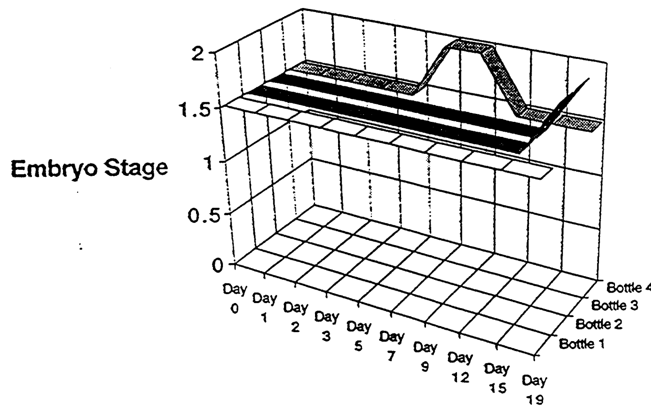


Figure 6. Dry weight of cells for three genotypes of Loblolly pine suspension cultures over a 19 day subculture. **A.** Genotype 71 **B.** Genotype 41 **C.** Genotype 195.

A. **Embryo Stage vs. Time: Genotype 71**



B. Embryo Stage vs. Time: Genotype 41



C. Embryo Stage vs. Time: Genotype 195

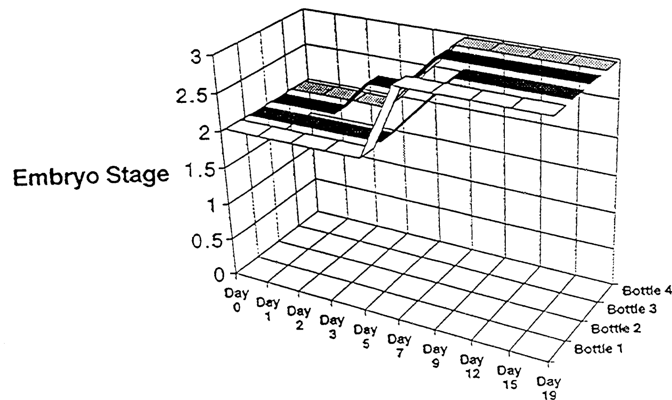


Figure 7. Embryo stage for three genotypes of Loblolly pine suspension cultures over a 19 day subculture. **A.** Genotype 71 **B.** Genotype 41 **C.** Genotype 195

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS: GERMINATION OF SOMATIC EMBRYOS

Gerald Pullman
Yolanda Powell
Shannon Johnson
Barbara Johns

March 21-22, 1995

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS: GERMINATION OF SOMATIC EMBRYOS

Gerald Pullman, Yolanda Powell, Shannon Johnson, and Barbara Johns

The Forest Biology Group previously reported the germination of Loblolly pine somatic embryos which exhibited both shoot and root growth *in vitro*. These somatic germinants (Figure 1) represent the first Loblolly pine plants from somatic embryos grown at the Institute.

Somatic embryos of genotype 195, produced in liquid maintenance medium 16 and harvested from development and maturation media 225 and 240, germinated on medium 55 (Table 1), a 1/2 MS basal salts medium containing 2.5 g/L activated charcoal. There has been limited success in germinating embryos of genotype 197.

During the first 4-5 weeks, the hypocotyl region of the embryo elongated followed by expansion of the cotyledons. In the following 4-5 weeks apical primordia appeared and slowly expanded into needle growth. After approximately two months root growth began.

While the above sequence of germination is slow and awkward when compared to natural seed embryos, the ability to produce somatic germinants repeatedly from one and possibly several genotypes provides us with a baseline for future improvement.

All of the cotyledonary somatic embryos used for germination resulted from early-stage embryos grown in liquid medium with subsequent growth and maturation on a semi-solid gelled medium. Since early-staged embryos in liquid culture show multiplication rates of 2-6 times weekly, the front end of this process is very efficient in rapidly producing large numbers of embryos.

Figure 1. Germinating somatic embryos of Loblolly Pine genotype 195. Note shoot and root growth.

Table 1. Composition of liquid culture maintenance medium (16), development and maturation media (225 and 240), and germination medium (55).

Components	Media (mg/l)			
	16	225	240	55
NH_4NO_3	603.8	--	200	206.3
KNO_3	909.9	100	909.9	1170
$MgSO_4 \bullet 7H_2O$	246.5	370	246.5	185
KH_2PO_4	136.1	170	136.1	85
$Ca(NO_3)_2 \bullet 4H_2O$	236.2	--	236.2	--
$Mg(NO_3)_2 \bullet 6H_2O$	256.5	--	256.5	--
$MgCl_2 \bullet 6H_2O$	101.7	--	101.7	--
KCl	--	745	--	--
$CaCl_2 \bullet 2H_2O$	--	440	--	220
KI	4.15	0.83	4.15	0.415
H_3BO_3	15.5	6.2	15.5	3.1
$MnSO_4 \bullet H_2O$	10.5	16.9	10.5	8.45
$ZnSO_4 \bullet 7H_2O$	14.4	8.6	14.4	4.3
$NaMoO_4 \bullet 2H_2O$	0.125	0.25	0.125	0.125
$CuSO_4 \bullet 5H_2O$	0.125	0.025	0.125	0.0125
$CoCl_2 \bullet 6H_2O$	0.125	0.025	0.125	0.0125
$FeSO_4 \bullet 7H_2O$	6.95	27.8	13.9	13.93
Na_2EDTA	9.33	37.3	18.65	18.63
Maltose	--	20,000	20,000	--
Sucrose	30,000	--	--	20,000
Polyethylene glycol (8,000 M.W.)	--	130,000	130,000	--
myo-Inositol	1,000	100	100	100
Casamino acids	500	--	500	--
L-Glutamine	450	1450	450	--
Thiamine•HCl	1.0	0.1	1.0	1.0
Pyridoxine•HCl	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5
Glycine	2.0	--	2.0	2.0
2,4-D	1.1	--	--	--
BAP	0.45	--	--	--
Kinetin	0.43	--	--	--
ABA	--	5.2	5.2	--
Activated charcoal	--	--	--	2,500
Hazelton Tissue Culture Agar	--	--	--	8,000
Gelrite	--	2,500	2,500	--
pH	5.7	5.8	5.7	5.7

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS: CONVERSION OF LOBLOLLY PINE SOMATIC EMBRYO GERMINANTS TO ESTABLISHED SEEDLINGS

Gerald Pullman
Shannon Johnson
Barbara Johns
Yolanda Powell

March 21-22, 1995

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS: CONVERSION OF LOBLOLLY PINE SOMATIC EMBRYO GERMINANTS TO ESTABLISHED SEEDLINGS

Jerry Pullman, Barbara Johns, Shannon Johnson, and Yolanda Powell

INTRODUCTION

The major goal of the Forest Biology Tissue Culture Group is to clone elite conifers through somatic embryogenesis. Improvement of embryo yield and quality has furthered our group's progress toward fulfillment of this goal. With the germination of somatic embryos the process of conversion to seedlings has begun.

A series of conversion trials are presented which progress from a closed sterile soil system to harsher open greenhouse systems. A general protocol for conversion has been developed, standardizing media preparation, and planting. Planting media, nutrient solutions, environment, light intensity, soil moisture evaporation, and the type of system the germinants are cultured in may influence the conversion process. A relationship between germinant quality and an individual's ability to survive conversion led to a concept called 'plantibility'.

During the conversion trials four distinct developmental stages began to emerge after planting: an acclimatization period, initial growth and greening of the epicotyl, epicotyl stabilization, and continued growth of the epicotyl. All germinants progressed through these stages regardless of parameters called for in individual research plans.

Future investigations will further define embryo and germinant quality to refine the conversion protocol and allow the Forest Biology Tissue Culture Group to direct research efforts toward a commercial greenhouse operation.

CONVERSION FACTORS

'Plantibility'

Presently the Forest Biology Group classifies somatic embryo development by morphological stage. No method of categorizing embryo quality (except 'normal' vs. 'abnormal') has been devised. The concept of 'plantibility' begins to address somatic embryo quality by describing the appearance of resulting germinants in comparison to zygotic seedlings. Germinants for conversion trials were derived from somatic embryos of genotype 195 plated on various maturation and development media from liquid suspension cultures. Only embryos which had formed a shoot of 1.0 mm or greater and a root were considered germinants. In screening germinants for the first conversion trials marked differences in appearance and amount of root and shoot growth were observed. Initially germinants were assigned to one of four groups based solely on hypocotyl appearance and root length and development. Roots were classified as minimal (less than 2.0 mm in length), moderate (2.0 mm-5.0 mm), and developed (greater than 5.0 mm). Developed and moderate roots

always showed some lateral root growth but minimal roots did not. These groups are subjective but attempt to delineate an individual's suitability for planting.

Group 1 germinants were extremely deformed with twisted or corkscrewed hypocotyls and minimal root growth. It was difficult to keep the root in contact with the planting medium without burying the shoot.

Group 2 germinants had less twisted hypocotyls with minimal or moderate root development.

Group 3 germinants formed a bent hypocotyl and a moderate or developed root.

The best looking germinants were assigned to Group 4 and appeared the most similar to zygotic seedlings with straight, elongated hypocotyls and developed roots.

Later experiments showed shoot growth might be of greater importance in conversion than root growth. After further trials the 'plantibility' groups were revised to include shoot development.

Planting Media

Three planting media were investigated: Sorbarod, a rolled paper fiber tube resembling a cigarette filter, Jiffy 7 peat pellet, and an artificial soil mix from Union Camp Corporation composed of peat moss, vermiculite, perlite, gypsum, and fertilizer. Sorbarods and Jiffy 7 peat pellets were used to successfully germinate excised loblolly pine zygotic embryos by Dr. David Webb (1).

Nutrient Solution

After planting, germinants were watered-in with Hoagland's #2 basal salt mixture (Sigma H2395) or 1/4 strength Schenk and Hildebrant basal salts (Table 1) and fertilized at regular intervals.

Environment

Autoclaved magenta boxes and media were employed for treatments in conversion trials requiring a sterile planting environment. All planting and maintenance operations were carried out in a laminar flow hood with sterile tools and disposable pipettes, and the boxes were wrapped with parafilm. In non-sterile treatments germinants were planted directly in magenta boxes or Styrofoam blocks of four 44 X 18 mm cells (Figure 1). Non-sterile vessels were washed, soaked in a 25% solution of bleach, and rinsed in water before use.

Light Intensity

Germinants grown under inadequate light intensity can develop a weak spindly epicotyl. In an effort to increase light intensity in the culture room the screens were removed from beneath the lights of several shelves. This resulted in an increase in light intensity from approximately 28 foot-candles to 48 foot-candles, a value roughly equal to a plant growth chamber utilized in some trials. Unlike the culture room and growth chamber, light intensity

Basal Salts	Hoagland's	SH
KNO_3	606.60	2500
$MgSO_4 \bullet 7H_2O$	240.76	400
$NH_4H_2PO_4$	115.03	300
$CaCl_2 \bullet 2H_2O$	-	200
$Ca(NO_3)_2$	656.40	-
$MnSO_4 \bullet H_2O$	-	10.0
$MnCl_2 \bullet 4H_2O$	1.810	-
H_3BO_3	2.860	5.0
$ZnSO_4 \bullet 7H_2O$	0.220	1.0
KI	-	1.0
$CuSO_4 \bullet 5H_2O$	0.080	0.2
$NaMoO_4 \bullet 2H_2O$	-	0.1
MoO_3	0.016	-
$CoCl_2 \bullet 6H_2O$	-	0.1
$Fe_2(C_4H_4O_6)_3$	5.320	-
$FeSO_4 \bullet 7H_2O$	-	15.0
Na_2EDTA	-	20.0
pH	5.0	5.4

Table 1: Nutrient Solutions of Schenk and Hildebrant and Hoagland's #2 Basal Salt Mixture

in the greenhouse is erratic (full sun vs. overcast) and the photoperiod could not be regulated precisely.

Temperature

The temperature in both the culture room and growth chamber was a constant 72° F. The temperature in the greenhouse can greatly fluctuate from day to day and through the night/day cycle.

Soil Moisture Evaporation

Early conversion trials using plastic corsage boxes as containers for styrofoam blocks suffered a loss of humidity and soil moisture. This was particularly severe in the plant growth chamber where air flow was strong. Closing the system and adding a moistened polyester batting pad corrected the problem. Little moisture loss has been noted from sealed boxes but boxes with Suncaps (Sigma C6920) lose approximately 2.0 g through evaporation per week. Several trials experimented with methods for maintaining high humidity within the open system.

Systems

In a closed system germinants were planted in magenta boxes and placed in the culture room. This system provided for fairly stable humidity and soil moisture. However since no drainage from the soil occurs in a closed system salt accumulation is a potential problem.

Open systems were more often used in the greenhouse (and plant growth chamber) in an attempt to set up protocols more closely resembling a commercial project. This included taped corsage boxes, a mist bed and a humidifier tent.

GENERAL PROTOCOL FOR CONVERSION

Through a series of experiments, a general protocol has developed to facilitate conversion of somatic germinants to seedlings by standardizing medium preparation and planting.

Planting Medium Preparation

The best planting medium investigated to date is the artificial soil mix. This mix, provided by Union Camp was easy to plant in and holds moisture well. Twenty-five grams of artificial soil mix was hydrated with 25.0 ml of double distilled water. "Hydrated" was defined as the amount of liquid needed to reach soil plasticity where soil plasticity is demonstrated as the ability of the artificial soil mix to be extremely compressed and hold the shape without expressing excess liquid. Thus hydrated, the 25.0 g of artificial soil mix and 25.0 ml weighs approximately 50.0 g.

The hydrated artificial soil mix was put into a magenta box and gently tamped down with a 35.0 cm square bar. For trials with sterile environments the boxes were autoclaved at 121° C for 20 minutes and aired overnight in a

laminar flow hood. This dissipated any toxic vapors created by autoclaving the peat based soil mix. However the soil mix dried out to several centimeters below the surface and required re-hydration with 25-30 ml of double distilled water.

Non-sterile styrofoam cells were first loosely filled with hydrated soil mix and a thin plug was made in the bottom of the open cell by compressing the soil mix with a 15.0 mm bore rod. This allowed for drainage without soil loss. The cells were again loosely filled with soil mix and gently tamped down. To avoid excessive settling of the soil mix, this was repeated several times until the soil surface was level with the top of the cell. The soil mix was saturated with water before use.

Planting Germinants

Somatic embryos were harvested from maturation and development media and transferred to germination medium (see Germination section in this report). The embryos were placed in the light culture room after a seven day period in the dark and allowed to germinate. The germinants were rated and collected in sterile petri plates on a moistened filter paper.

Since most germinants had short roots, a small shallow hole was made in the surface of the soil mix and the germinant was inserted with forceps. The soil mix was tamped around the base of the hypocotyl and the germinant was watered in with the appropriate nutrient solution as previously stated. Magenta boxes were capped with an opaque lid or covered with a Suncap, a transparent polypropylene film. For some trials styrofoam blocks were placed in clear plastic corsage boxes on a moistened polyester batting pad and the box was taped closed.

Hypocotyl and initial epicotyl measurements are recorded.

Cultural Practices

After planting the magenta boxes and styrofoam blocks were placed in the appropriate location (light culture room, plant growth chamber or greenhouse). Epicotyl measurements and observations on epicotyl appearance and soil condition were recorded weekly. Germinants in styrofoam blocks were watered or fertilized each week and polyester batting pads in corsage boxes were rinsed and moistened every four days. Germinants in magenta boxes were fertilized according to soil moisture.

CONVERSION DEVELOPMENTAL STAGES

Four developmental stages emerged from regular observations made on germinants after planting. At first germinants appeared to spend several weeks in an acclimatization period. The germinants were a dull dusty green color and no growth of the epicotyl occurred nor was any further extension of the hypocotyl noted.

After two to four weeks new epicotyl growth began to appear as bright shiny needles growing upward out of the initial shoot. This burst of growth might last for a week or two. The hypocotyl showed no increase in length.

Following acclimatization and initial growth, the epicotyl ceased to grow. The germinant maintained its vigor and the new epicotyl growth remained bright green and the needles spread out. Some germinants are still in this condition while others completed conversion.

The establishment of a germinant as a somatic seedling was successful when the seedling began actively growing again. These seedlings were transplanted into leach tubes. At present we have approximately 42 converted somatic seedlings (Figure 2). they are housed in the culture room under high humidity and will be opened to the ambient environment soon.

DISCUSSION AND CONCLUSIONS

Conversion of Loblolly pine somatic embryo germinants to established seedlings was achieved in the four completed trials with percent mortality recorded for one ongoing trial. Objectively there was no statistical differences found between treatments within trials for percent conversion or percent mortality with one exception; a closed vs. open system. The closed system showed a higher percent conversion and a lower percent mortality. This may result from maintaining a high humidity resulting in little soil moisture loss. With a high humidity in non-sterile closed systems, contamination by plant pathogenic organisms was expected to be a problem. However, only one plant was lost to fungus.

Although little statistical correlation can be made between conversion and nutrient solutions, the 1/4 Schenk and Hildebrant basal salts produced comparatively more seedlings with epicotyl height of 7.0 mm or greater than did those germinants fertilized with Hoagland's.

The following tables illustrate conversion for four trials and mortality for a fifth on-going trial.

Conversion and Mortality in Trials

Trial 1

Treatment	Conversion
1	1/11 (9%)
2	1/11 (9%)
3	0/11 (0%)
4	1/11 (9%)

Treatment 1: Hoagland's nutrient solution, Sorbarod planting medium, closed system, sterile environment, culture room.
Treatment 2: Hoagland's, Jiffy 7 peat pellet planting medium, closed system, sterile environment, culture room
Treatment 3: Hoagland's, Union Camp artificial soil mix, closed system, sterile environment, culture room
Treatment 4: 1/4 Schenk and Hildebrant nutrient solution, Union Camp artificial soil mix, closed system, sterile environment, culture room.

Trial 2

Treatment	Conversion
1	0/12 (0%)
2	2/12 (17%)
3	0/12 (0%)
4	0/12 (0%)

Treatment 1: Hoagland's, UC soil mix, open, non-sterile environment, greenhouse
Treatment 2: 1/4 SH, UC soil mix, open, non-sterile environment, greenhouse
Treatment 3: Hoagland's, UC soil mix, open, non-sterile environment, plant growth chamber
Treatment 4: 1/4 SH, UC soil mix, open, non-sterile environment, plant growth chamber.

Trial 3

Treatment	Conversion
1	12/16 (75%)
2	6/16 (37%)

Treatment 1: 1/4 SH, UC soil mix, closed, sterile, culture room
Treatment 2: 1/4 SH, UC soil mix, closed non-sterile, culture room.

Trial 4

Treatment	Conversion
1	3/12 (25%)
2	4/12 (33%)
3	5/12 (42%)
4	6/12 (50%)

Treatment 1: Hoagland's, UC soil mix, open, non-sterile, greenhouse
Treatment 2: 1/4 SH, UC soil mix, open, non-sterile, greenhouse
Treatment 3: Hoagland's, UC soil mix, open, non-sterile, plant growth chamber
Treatment 4: 1/4 SH, UC soil mix, open, non-sterile, plant growth chamber.

Trial 5

Treatment	Extant Germinants	Mortality
1	5/16 (31%)	11/16 (69%)
2	8/16 (50%)	8/16 (50%)
3	14/16 (87%)	2/16 (12.5%)

Treatment 1: 1/4 SH, UC soil mix, open, non-sterile, greenhouse/mist bed
Treatment 2: 1/4 SH, UC soil mix, open, non-sterile, greenhouse/humidifier
Treatment 3: 1/4 SH, UC soil mix, closed, sterile, culture room.

Epicotyl Growth in Seedlings (Trials 1-4)

		Height	Height
Nutrient Soln	System	6.0 mm or less	7.0 mm or greater
1/4 SH	Open	10/31	2/31
	Closed	10/31	10/31
Hoagland's	Open	7/9	0/9
	Closed	1/9	1/9

Observations made throughout the trials led to several subjective conclusions. The most outstanding was germinant quality. Poor quality germinants had a very low survival rate and none have achieved conversion. As the trials progressed the quality of germinants available for planting improved and so did survival and conversion rates, particularly in a closed system.

The closed system regardless of growing container (magenta box, or taped corsage box) or test site (culture room, greenhouse, or plant growth chamber) resulted in the greatest percentage of germinants reaching conversion. A

closed system reduces soil moisture evaporation and maintains a high humidity, both which were found to be very important.

FUTURE EXPERIMENTS

Further trials are needed to investigate the factors controlling conversion and incorporate them into our present protocol. We can begin by rating somatic embryos transferred from ESM on maturation and development media to germination medium and track the resulting germinant and seedling quality and survival. This may lead us to a good quality control system for culling inferior embryos. Embryos on germination medium could be placed under a higher light intensity than our current protocol calls for.

The artificial soil mix used in these trials has been amended with fertilizer which could be eliminated to test nutrient solutions that promote better shoot and root growth.

Vessels such as magenta boxes should be replaced in favor of styrofoam blocks with larger cells than were used in these trials to allow proper root growth and provide a greater volume of growing medium to prevent the leaching of nutrients.

These observations and conversion results demonstrate that Loblolly pine somatic embryos can be converted repeatedly to somatic seedlings in several environments ranging in severity. The successful conversion in a greenhouse humidifier/plastic tent environment provides optimism for future success of low cost greenhouse systems suitable for commercial production.

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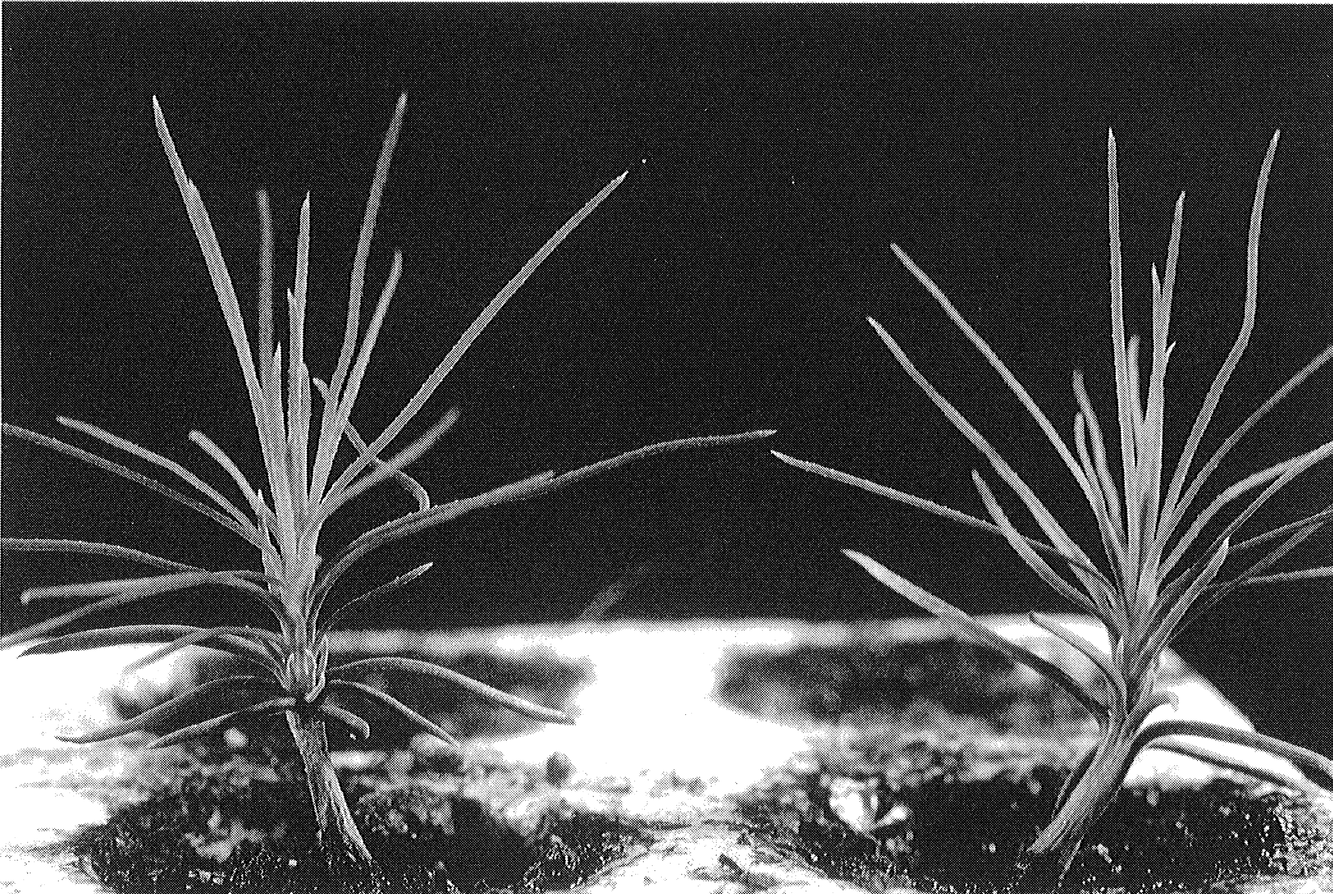

Figure 1. Converted somatic seedlings of genotype 195 growing in styrofoam cells. Styrofoam cells were used in open system non-sterile conversion trials. Seedlings were transferred to leach tubes.

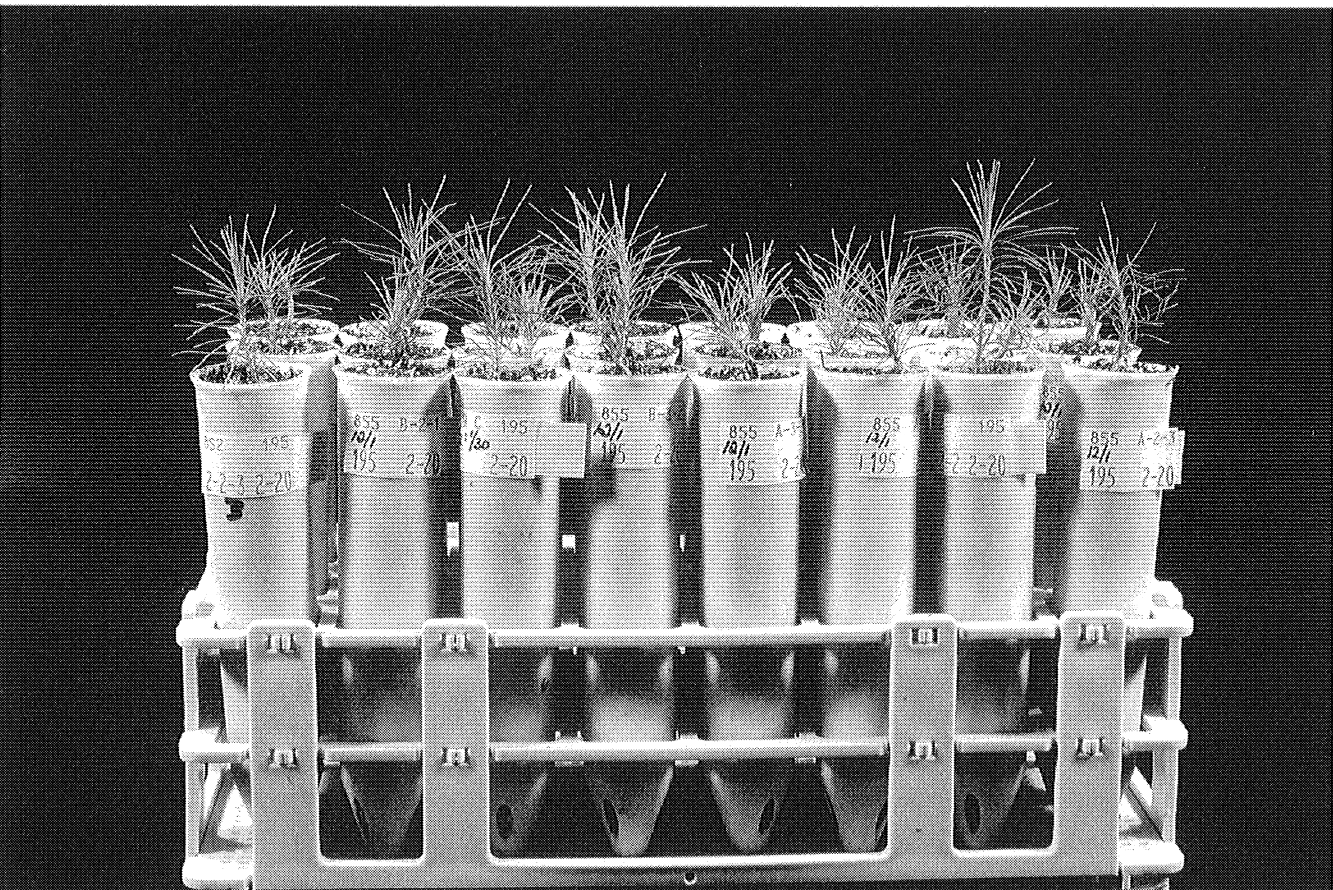

Figure 2. Converted somatic seedlings of genotype 195 growing in leach tubes. These seedlings are derived from several conversion trials.

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

Osmotic Measurements During Zygotic Embryogenesis in Loblolly Pine

Gerald Pullman
Greg Eley

March 21-22, 1995

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The nutritional and hormonal environment surrounding an embryo is well known to control embryo growth. These nutritional and hormonal components create an additional environment when dissolved in water, the osmotic environment. The osmotic environment has been shown, both in angiosperms and gymnosperms, to play an important role in altering embryo development, particularly in preventing precocious germination. No information on the osmotic environment during loblolly pine embryo development is currently available in the literature. Thus, most loblolly pine somatic embryogenesis trials rely on empirical experiments to determine the optimal media components.

IPST experiments with liquid suspension cultures of loblolly have demonstrated that the osmotic environment changes due to autoclaving and culture growth (March 21, 1994 Forest Biology Project Advisory Committee Status Report). In addition, maltose and sucrose create different osmotic environments during early-stage embryo development and maturation. The use of maltose is the subject of IPST U.S. Patent #5,187,092 for improved embryo development.

Work was begun in the summer of 1993 to understand the osmotic environment during loblolly pine zygotic embryo development. Loblolly pine cones were collected weekly from Boise Cascade and Union Camp breeding orchards and shipped on ice to IPST. Cone were received within 24-48 hours of collection. Initial experiments showed that ovules and embryos dried rapidly during excision, therefore, all opening of seeds and isolation of female gametophytes was done in a moist chamber. A moist chamber which housed a dissecting scope was created by partially lining a transparent plastic box with wet paper towels. Two entry ports were covered with plastic flaps which allowed hands to enter the box for dissections under magnification. Initial isolations of embryos from the female gametophyte also showed fluids leaking from the cut surfaces. Since water potential is composed of solute or osmotic potential, matric potential, and turgor pressure components, it was feared that cut ovule surfaces would cause the solute potential to be measured rather than the water potential. It was decided, therefore, to measure the water potential of the whole ovule containing the female gametophyte and embryo. This is also based on the assumption that we are most interested in the osmotic environment surrounding the developing embryo and therefore should be reflected by the osmotic level of the whole structure.

A Wescor 5500 Vapor Pressure Osmometer was fitted with a 7 mm diameter x 2.5 mm deep sample holding pan, and modified with a cycle hold switch which allowed long-

term sample equilibration. Three to five whole loblolly pine ovules were removed from the seed coat and integuments and rapidly placed in the osmometer holding pan. Samples were allowed to equilibrate for 30 minutes and two osmotic measurements were taken. If osmotic values were not within 10 mmol/Kg of each other additional equilibration was allowed until two similar consecutive readings were obtained. In most cases a 30 minute equilibration period was sufficient to obtain consistent osmotic readings. This process was repeated for four cones from each collection time and mother tree. After osmotic readings were obtained, each ovule was opened and the embryo was rated for embryo stage according to the rating system described in previous PAC Reports (March 21, 1994).

In order to obtain ovule fresh weights, five whole ovules (female gametophyte and embryo) were isolated in the moist chamber, enclosed in a small pre-weighed aluminum weighing container, and weighed on a five place Mettler balance. Containers and embryos were dried overnight at 70 C and re-weighed to obtain dry weights. Data was obtained from embryos from each of four cones for each collection time and mother tree. From this data % water content, ovule fresh weight and ovule dry weights could be calculated.

1993 Results

Cones were received weekly from the Boise Cascade seed orchard near Lake Charles, Louisiana (Tree BC-1) and from the Union Camp seed orchard at Rincon, GA (Trees UC-10-1003 and UC-10-1015). Early season measurements for all cone collections showed high osmotic levels of 400-550 mmol/Kg (Figure 1a-c). To be sure that these high levels were not an artifact of drying during shipping, a local loblolly pine tree was chosen with cones that could be reached with a tree pole pruner. This tree, labeled "wild", was used to collect three cones weekly until no more cones could be reached. The "wild" tree cones were collected early in the morning (~ 7 am) and rushed to the lab on ice for osmotic measurements. Osmotic measurements from the "wild" tree cones showed similar high osmotic levels during early embryo development (Figure 1d), indicating that the shipping period was not causing erroneous measurements.

All four cone collections showed a consistent pattern of high osmolality of 400-550 mmol/Kg during early embryo development in late June to early July when embryo stages 1-2 occurred. All cone collections then showed a reduction in osmolality for one to several weeks (Figure 1 a-d). BC-1, UC-10-1003, UC-10-1015 and "Wild" reached low osmotic levels of 256, 334, 318, and 200 mmol/Kg respectively. In all collections the decrease in osmolality correlated with embryo stages 4 and 5 (Figure 2a-d). All cone collections except "wild", which ran out of reachable cones in mid-season, then showed a steady increase in osmotic measurements from late August onwards. From late August onwards osmotic measurements increased from approximately 350 to 700-800 mmol/Kg where they leveled off until cone harvest.

Embryo stage showed a lag for several weeks at stage one during mid to late June and early July (Figure 3 a-d). Observations of embryos showed that stage one embryos cleaved due to cleavage polyembryogeny followed by suspensor elongation. This repeated several times with each new set of stage one embryos located farther from the micropylar end until embryos reached a mid point within the ovule. From then on one dominent embryo progressed rapidly through stages 2-8 within 4-5 weeks. Stages 4-6 correlated well with the observed "valley of osmolality". Embryos then remained morphologically at stage nine but continued increasing in size and weight for an additional 6-8 weeks while osmolality steadily increased and leveled off at 700-900 mmol/Kg.

Percent water content of the whole ovule is shown in Figure 4 a-d. Percent water content showed a sigmoidal curve shape with a short lag period at approximately 90% water content as stage 1 persisted. A linear decrease in water content then occurred over most of embryo development. Approximate water content by embryo stage is shown in Table 1. Water contents by stage were very similar for all four tree collections. All embryos continued to accumulate fresh and dry weight while decreasing water contents. Over a period of 6-8 weeks, stage 9 embryos continued to increase in size and weight while osmolality varied from 350 to 900 mmol/Kg and average water content varied from 38-26% at maturity.

Table 1. Whole ovule percent water content by embryo stage.

Embryo Stage	BC-1	UC10-1003	UC10-1015	Wild	Average
1	90%		89%		89%
2	80%	73%	84%	80%	79%
3	75%	68 %	68%	74%	71%
4	73%	63%	59%	63%	65%
5	68%	59%	55%		61%
6	62%	57%	51%		57%
7	56%	54%	38%		49%
8	48%	46%	37%		44%
9 - Wk 1	39%	38%	38%		38%
9 - Wk 2	34%	34%	34%		34%
9 - Wk 3	34%	31%	33%		33%
9 - Wk 4	28%	28%	30%		29%
9 - Wk 5	26%	28%	26%		27%
9 - Wk 6	26%	22%	21%		23%
9 - Wk 7	26%	25%	26%		26%
9 - Wk 8	23%				

Fresh and dry weight accumulation throughout embryo development are shown in Figure 5 a-d. Fresh and dry weight also showed sigmoidal curves with a short lag, a linear increase, and a plateau at maturity. Fresh weight per ovule was consistently 2-4 mg greater than dry weight. The difference between fresh and dry weight ranged towards the

higher values for the larger seeded collections such as BC-1 and UC-10-1051. UC-10-1051 was small seeded and the wild collection cones were especially large seeded. The difference between fresh and dry weight remained fairly constant over the entire season. This indicates that a constant amount of water was present in the ovule regardless of the % water contents. Dry weights per ovule started around 0.5-1.0 mg and ended at 6, 8, and 13 mg for UC10-1003, UC10-1015, and BC-1 respectively. It is interesting to note that during the weeks of September, when osmolality rose above 500-600 mmol/Kg, embryo fresh and dry weight continued to increase.

Results 1994

During 1994 the above study was repeated. The same Boise Cascade tree, BC-1, from the same seed orchard near Lake Charles, Louisiana, was again used for cone collections. However, the Union Camp trees used in 1993 were not available for collection. Trees, UC-5-1036 and UC-7-1051, collected from a seed orchard near Bellville, GA were substituted. Weekly collections were made and shipped on ice as described for 1993. The same procedures for osmotic determinations, embryo stage, and fresh and dry weight were followed. In addition, three cones of BC-1 and UC-5-1036 each were used to isolate 10-20 embryos and female gametophytes per cone for fresh and dry weight determinations.

Osmolality measurements for whole ovules of BC-1 showed the same pattern of osmotic change through embryo development as was measured during 1993 (Figure 6a). The osmolality curve for UC-5-1036 showed a early declining osmotic level followed by a rise, a level region and then the late development osmotic rise (Figure 6b). The osmolality curve for UC-7-1051 showed a slightly different pattern than was measured in other tree collections during 1993 or 1994, a slowly rising pattern was observed starting from about 325 mmol/Kg (Figure 6c). For both tree collections UC-5-1036 and UC-7-1051 collections began on July 5, 1994 and missed the 2-3 weeks of stage 1.

Patterns of development for 1994 embryo stage, percent water content, and fresh and dry weights all showed similar patterns of change through the season as was seen in developing embryos during 1993 (Figures 7 a-c, 8 a-c, 9 a-c).

Both BC-1 and UC-10-1036 showed the decline in osmotic levels around embryo stage 4 similar to 1993 observations (figure 10 a-b). However, UC-7-1051 showed an osmotic increase during stages 4-5 (figure 10 c). Out of seven tree collections followed during 1993-1994, UC-7-1051 was the only one to show a osmotic rise during stages 4-5.

Embryo and female gametophyte fresh and dry weights are shown in Figure 11 a-d. Embryo fresh and dry weights showed a typical sigmoidal pattern of weight accumulation with the greatest mass accumulation during stages 8 and the first two weeks of stage 9. Note that in all cone collections, stages 8-9.2 were correlated with osmolality readings of 300-500 mmol/Kg. During the last four weeks of measurements, UC-5-1036 embryo

weights leveled off and BC-1 increased slightly. Female gametophytes showed a similar pattern of weight accumulation with the greatest mass accumulation occurring when embryos were at stages 7 through the fourth week of stage nine.

Conclusions

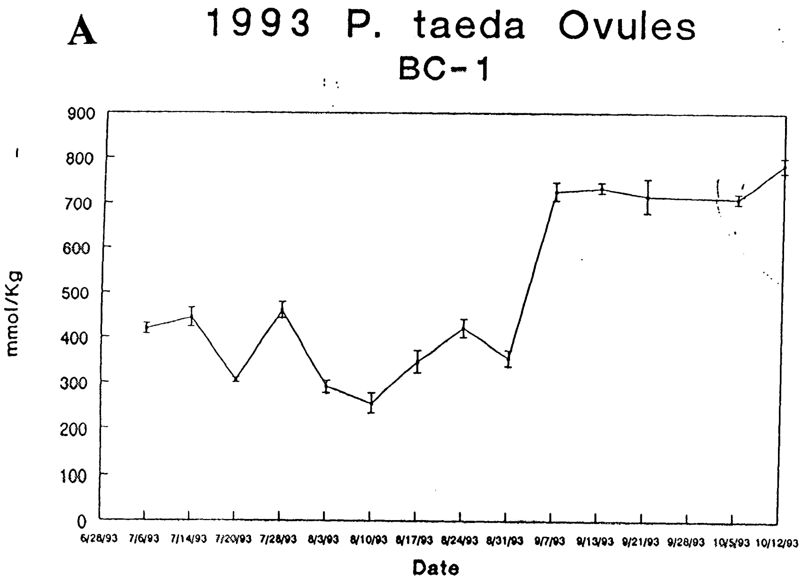
Two years of osmotic evaluations during loblolly pine embryo development have provided some striking information to guide us in somatic embryogenesis. 1) Osmotic levels start high, drop, and then increase. This pattern can be found in other developing seeds but is unusual. 2) The drop in osmotic levels gives us some insight as to why maltose provides an optimal maturation environment for loblolly pine. Sucrose breaks down in the medium into glucose and fructose during embryo growth thus increasing the osmotic environment. Maltose does not increase osmolality of the medium, so apparently it breaks down within the cell into two glucose molecules. Thus, the use of sucrose causes osmolality of the embryonic environment to rise while maltose allows osmotic levels to remain static or slowly decline. Maltose more closely resembles the natural osmotic profile. 3) The osmotic environment decrease usually occurred when embryos developed to stages 4-5. This is the embryo stage when the apical dome begins to form. A drop in osmotic stress may be critical for apical dome formation. 4) The greatest fresh and dry embryo weight accumulation occurred during embryo stages 8-9.2 when osmolality measured 300-500 mmol/Kg. Mass accumulation of embryo and female gametophyte continued to increase during late embryo development, when osmotic levels remained above 500 mmol/Kg.

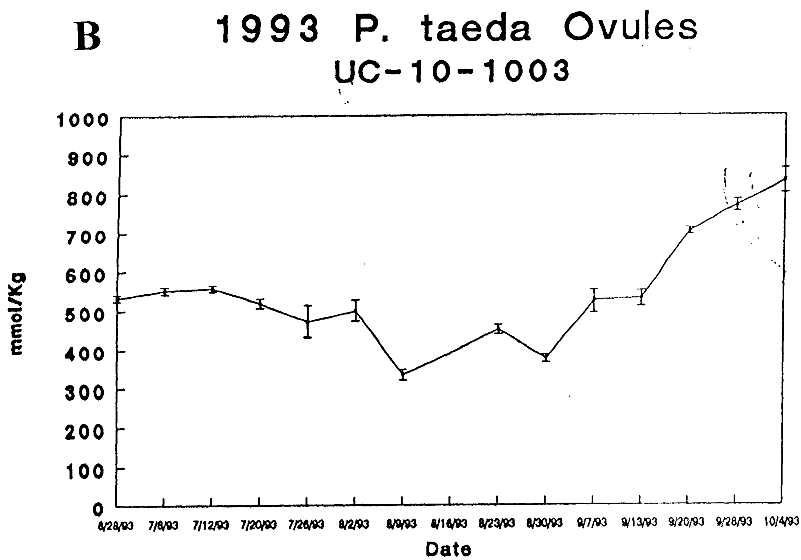
Gates and Greenwood (Gates, J. C. and M. S. Greenwood. 1991. The physical and chemical environment of the developing embryo of *Pinus resinosa*. Am. J. Bot. 78: 1002-1009) reported that osmotic potential increased slightly between the zygotic and proembryo stages and then remained constant. Our data are for water potential and more correctly measure the osmotic environment. These findings have significant implications for somatic embryo development. They suggest several changes in our current protocol.

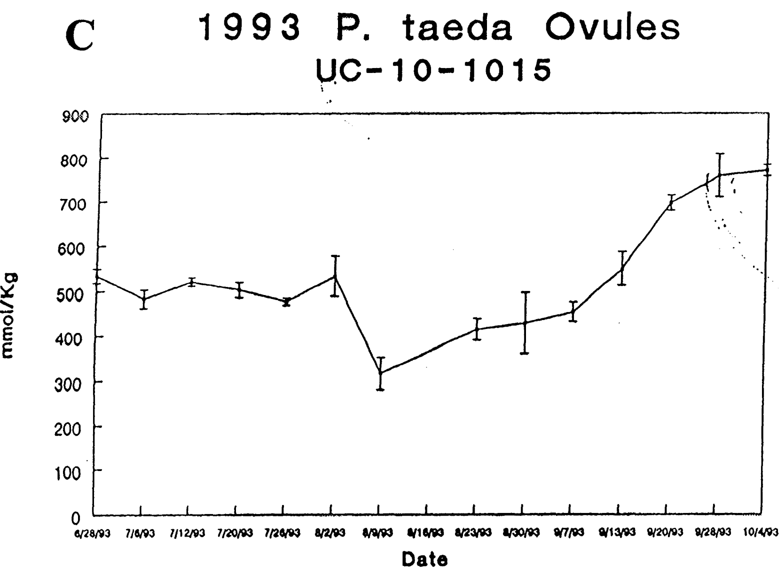
1) Initiate at high osmotic levels as opposed to standard osmolality levels of 100-160 mmol/Kg.
2) Maintenance media osmolality should target 300-500 mmol/Kg.
3) Development and maturation currently at a constant 250 mmol/kg should be increased to 300-400 mmol/kg with a decrease in osmoticant to 200-300 mmol/kg at stage 4-5.
4) Late embryo development medium, for stage 7 onwards should continuously increase to 500-700 mmol/Kg.

Although our initiation rates are low, we have already been successful in initiating loblolly pine embryogenic cultures at osmolalities above 200 mmol/kg. The above suggestions provide numerous hypotheses for testing which we are confident will lead to improvements in early and late stage embryo quality.

Figure 1. *Pinus taeda* whole ovule osmolality during 1993 embryo development as measured by a Wescor 5500 Vapor Pressure Osmometer. A. Cones from Boise Cascade BC-1. B. Cones from UC-10-1003. C. Cones from UC-10-1015. D. Cones from local Atlanta, GA Wild tree.







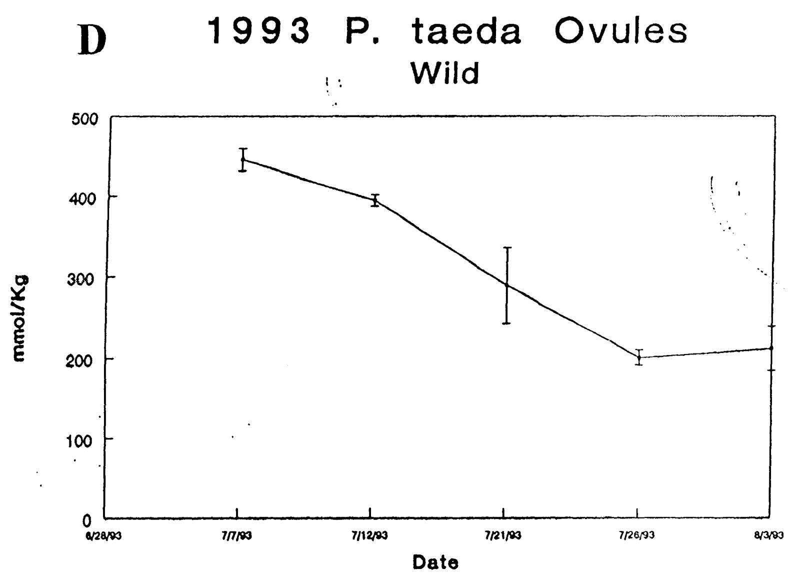


Figure 2. *Pinus taeda* whole ovule osmolality and embryo stage during 1993 embryo development. Note that as ovule osmolality begins to drop embryo development coincides with stages 4-5. A. Cones from Boise Cascade BC-1. B. Cones from UC-10-1003. C. Cones from UC-10-1015. D. Cones from local Atlanta, GA Wild tree.

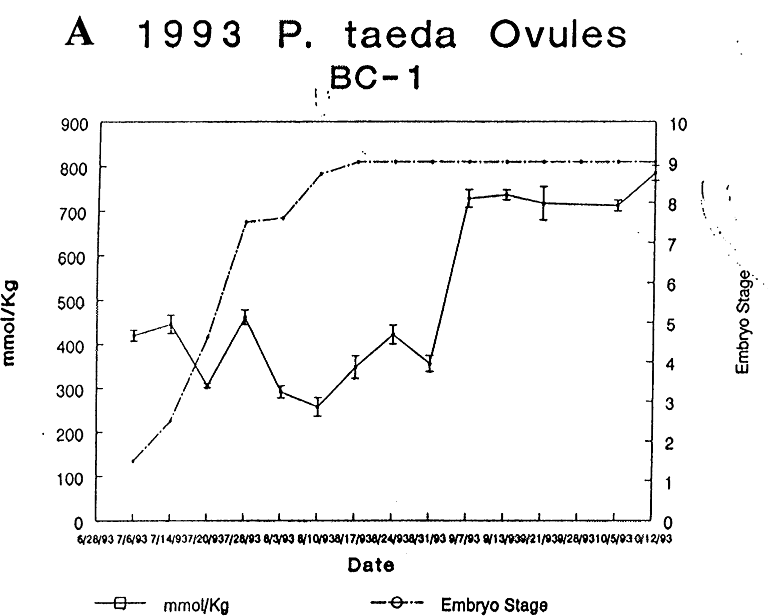
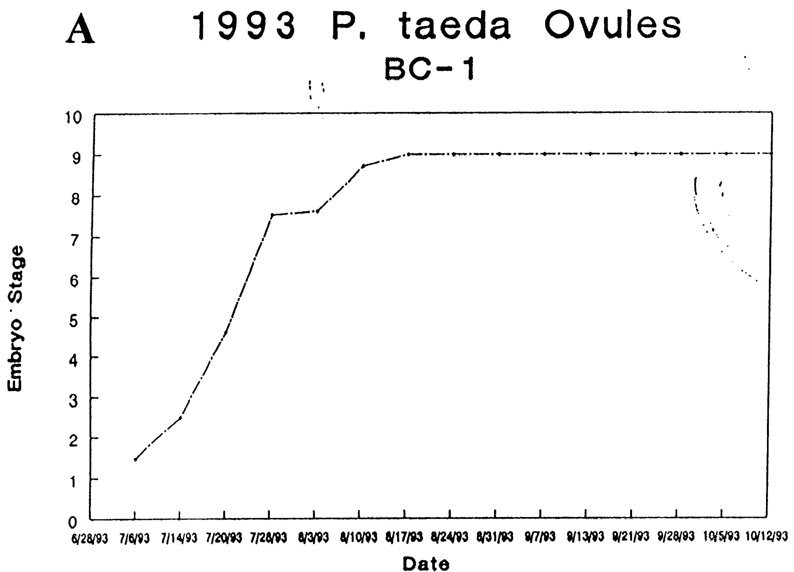
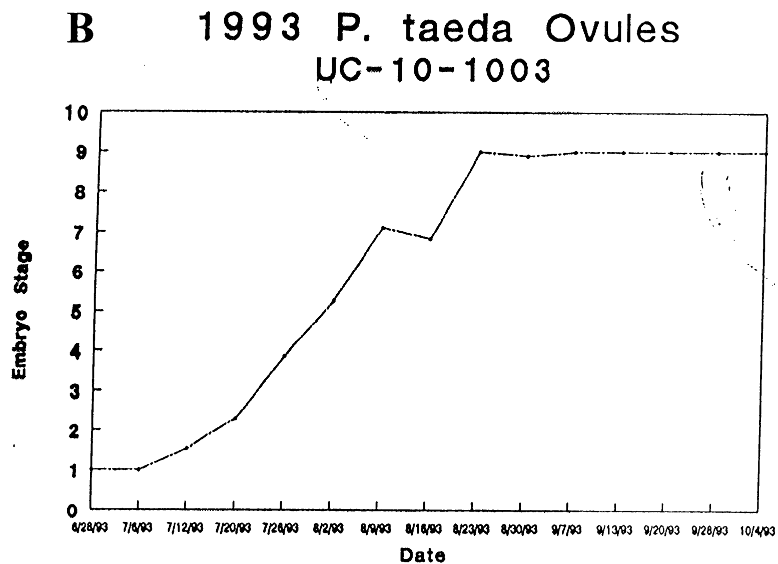
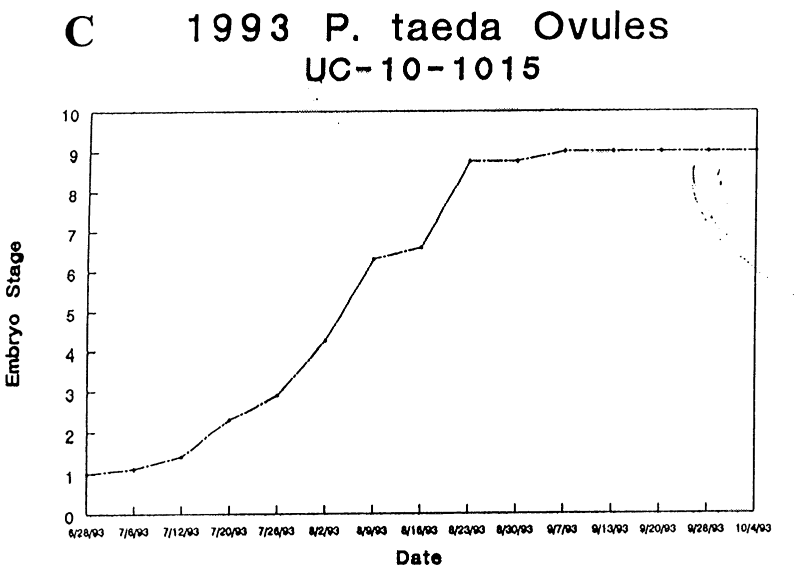


Figure 3. *Pinus taeda* embryo stage during 1993 embryo development. A. Cones from Boise Cascade BC-1. B. Cones from UC-10-1003. C. Cones from UC-10-1015. D. Cones from local Atlanta, GA Wild tree.







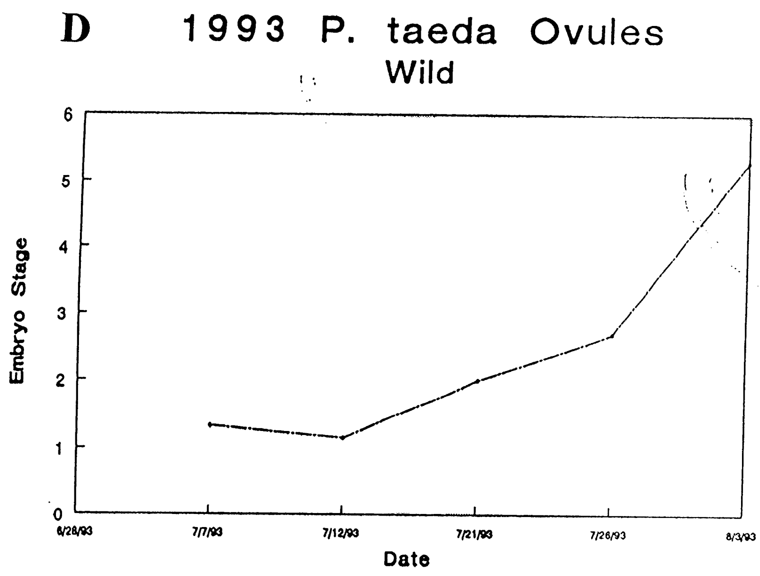
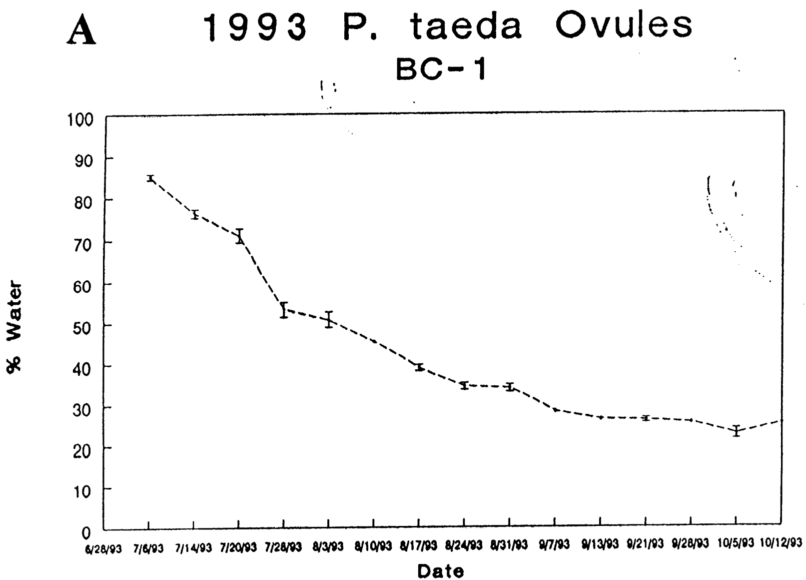
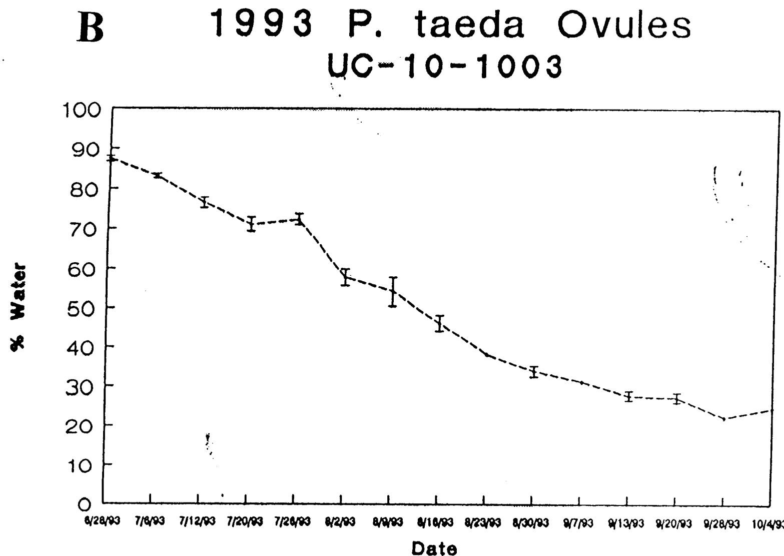
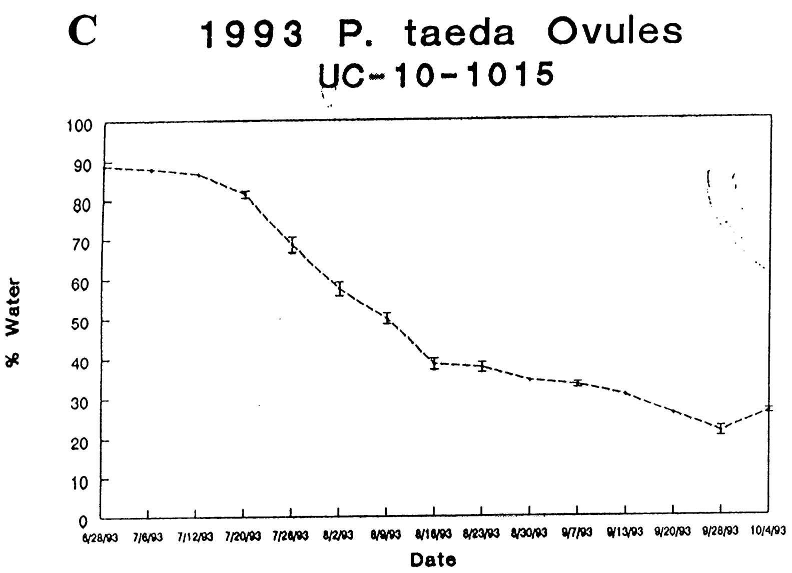


Figure 4. *Pinus taeda* percent water content of whole ovules 1993 during embryo development. A. Cones from Boise Cascade BC-1. B. Cones from UC-10-1003. C. Cones from UC-10-1015. D. Cones from local Atlanta, GA Wild tree.







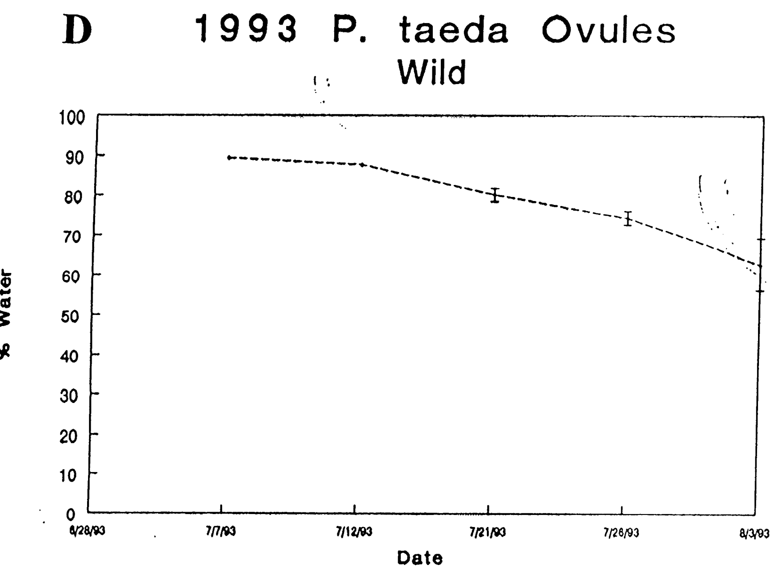
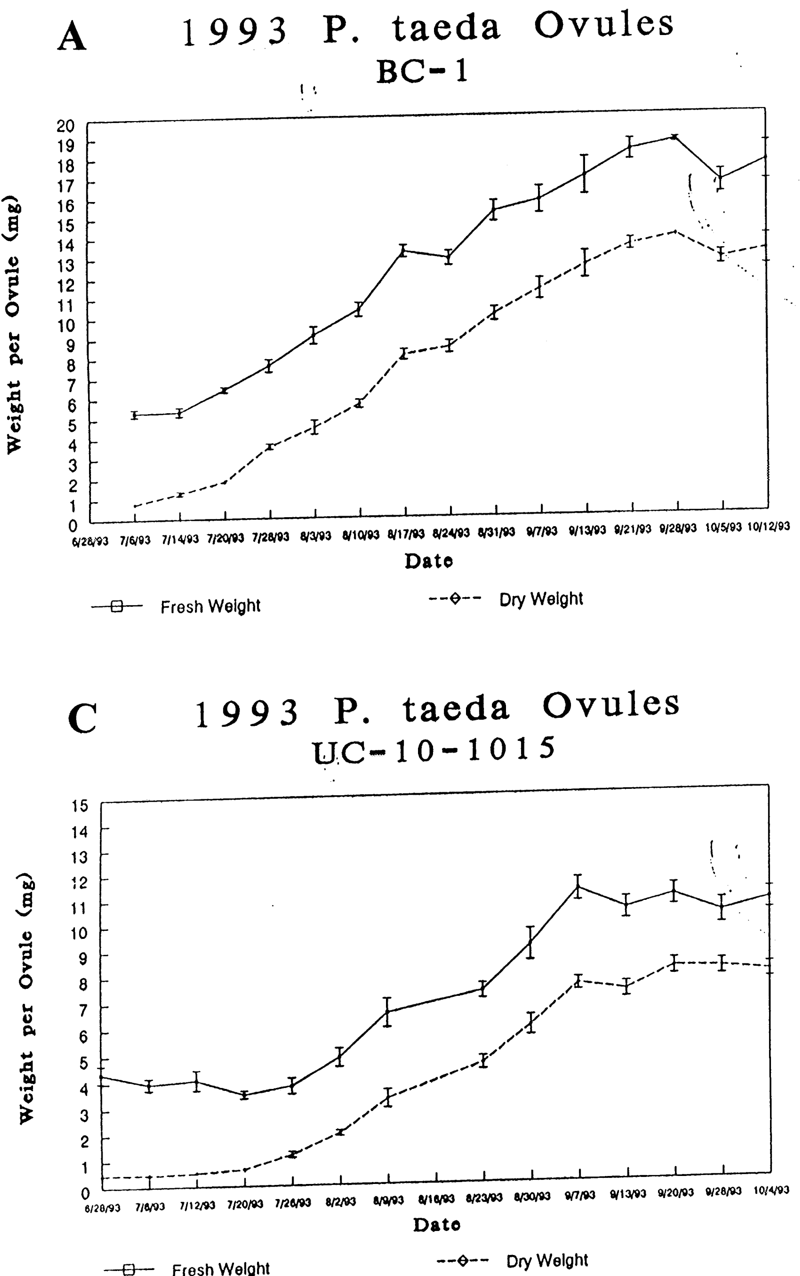


Figure 5. *Pinus taeda* whole ovule fresh and dry weight during 1993 embryo development. A. Cones from Boise Cascade BC-1. B. Cones from UC-10-1003. C. Cones from UC-10-1015. D. Cones from local Atlanta, GA Wild tree.



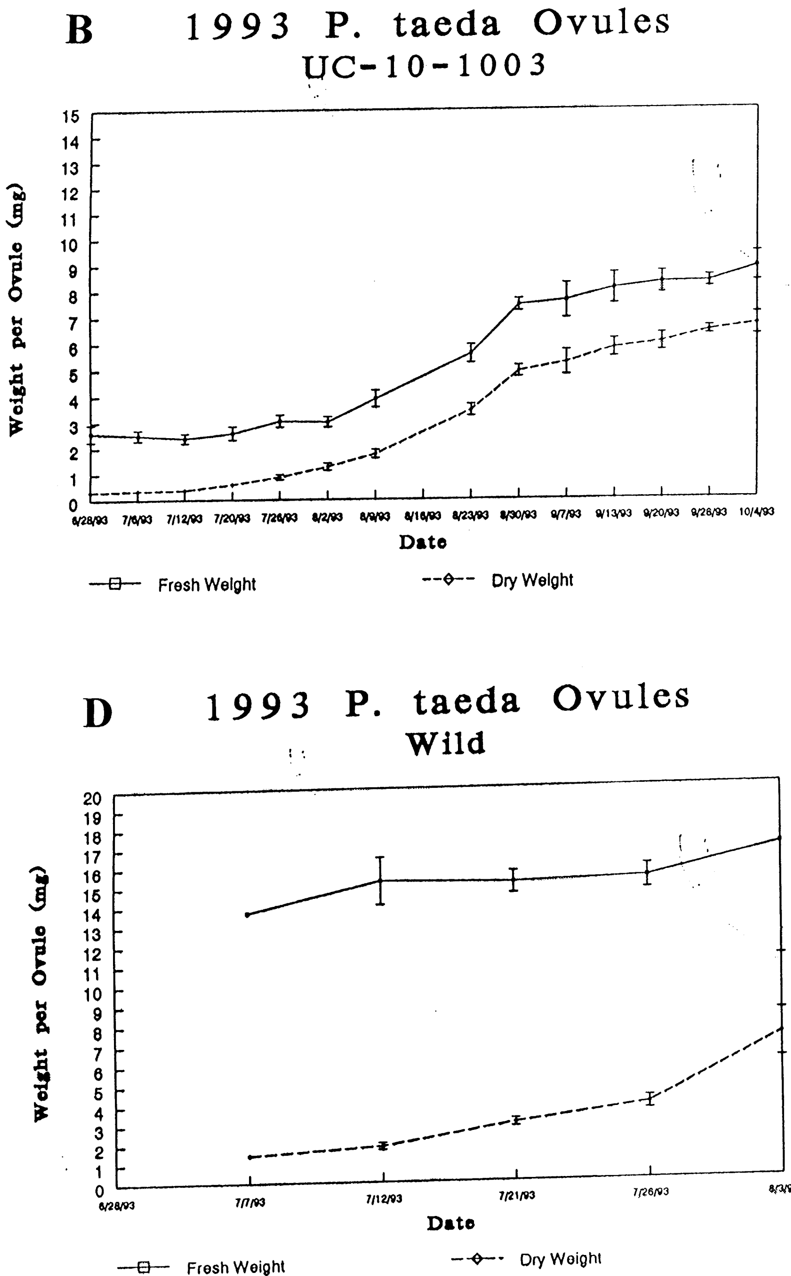
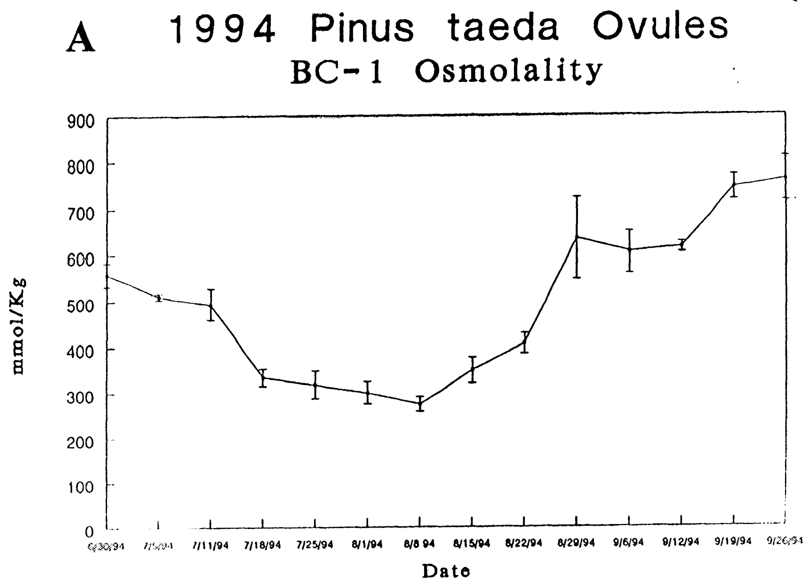
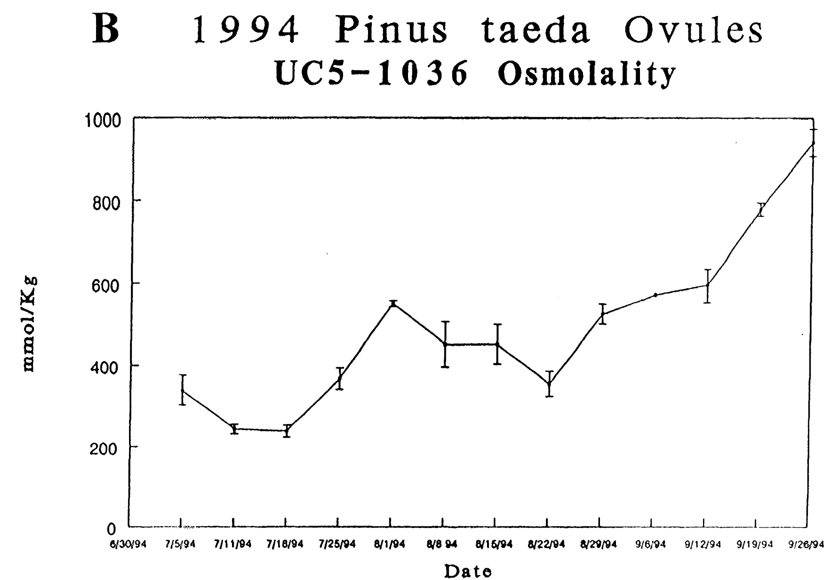


Figure 6. *Pinus taeda* whole ovule osmolality during 1994 embryo development as measured by a Wescor 5500 Vapor Pressure Osmometer. A. Cones from Boise Cascade BC-1. B. Cones from UC-5-1036. C. Cones from UC-7-1051.





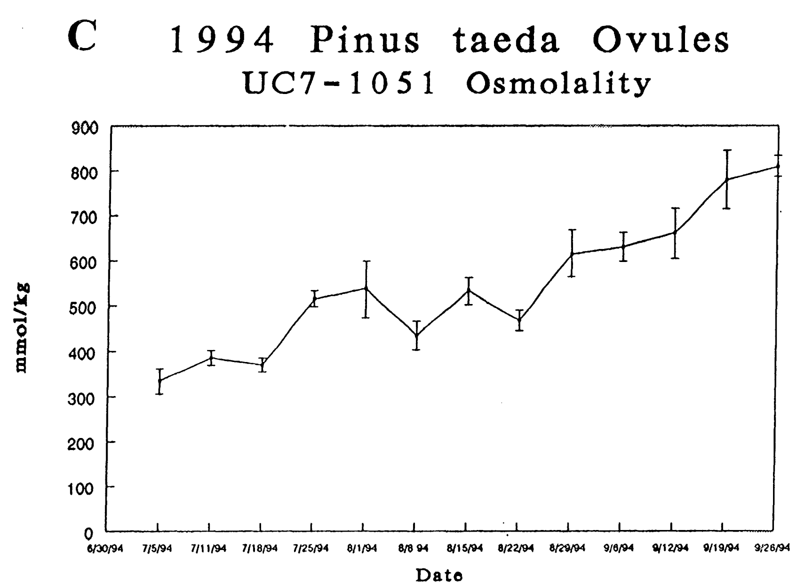


Figure 7. *Pinus taeda* embryo stage during 1994 embryo development. A. Cones from Boise Cascade BC-1. B. Cones from UC-5-1036. C. Cones from UC-7-1051.

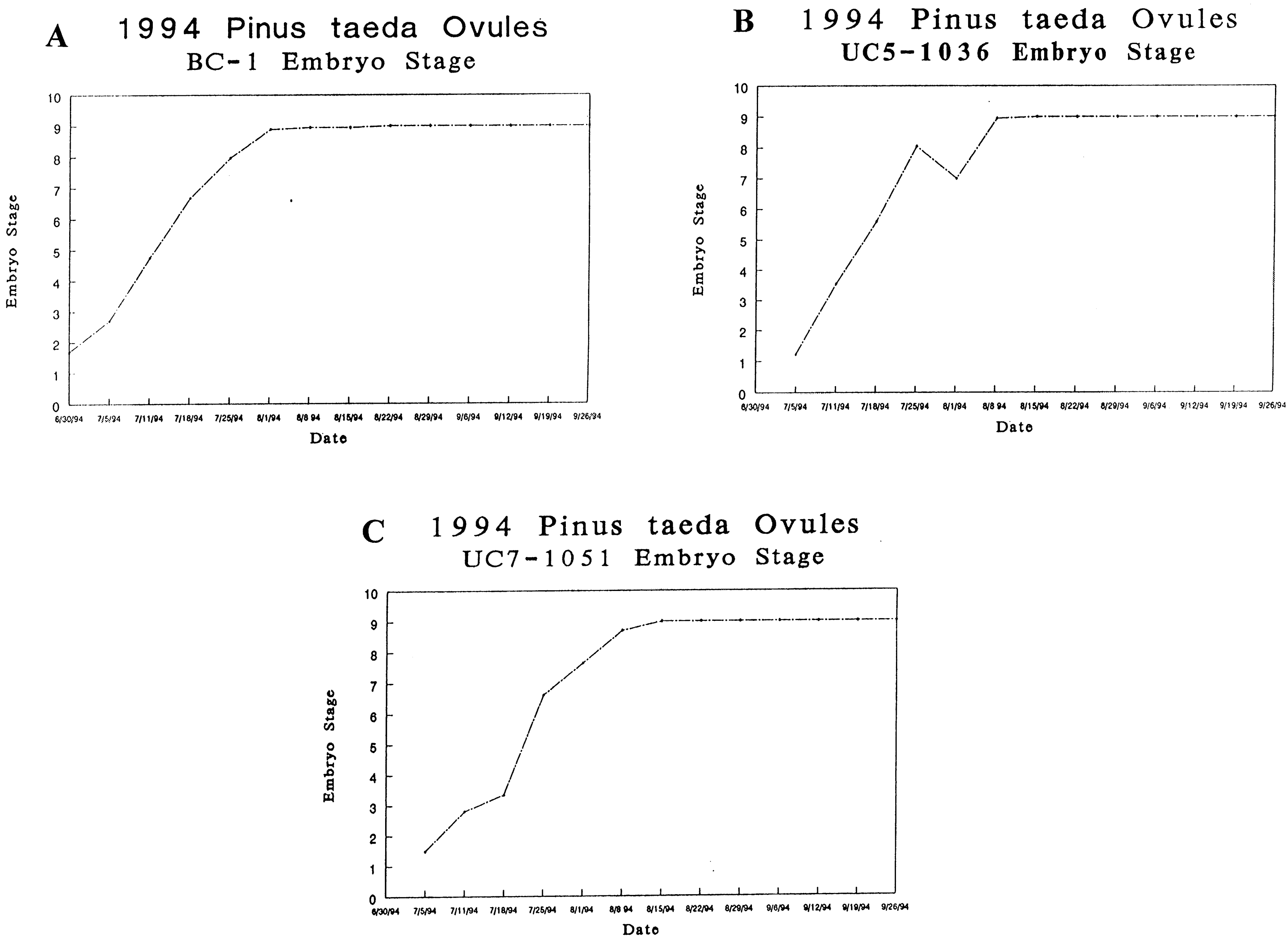
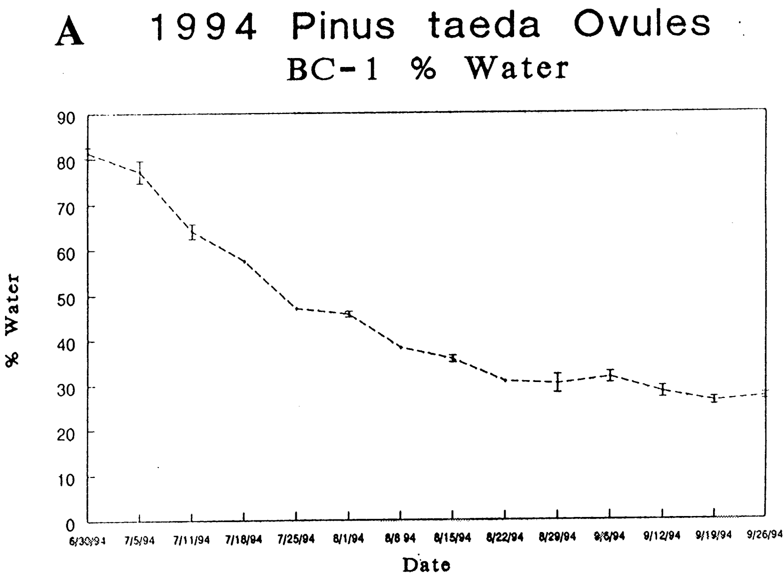
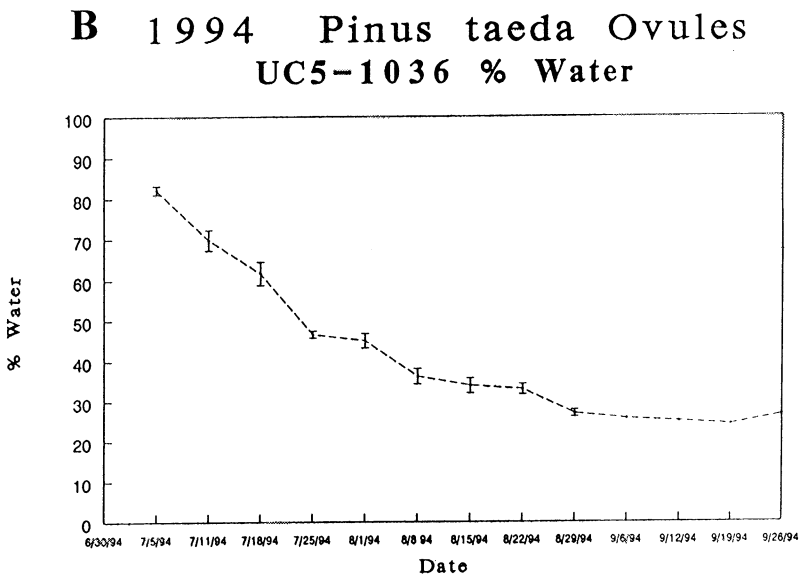


Figure 8. *Pinus taeda* percent water content of whole ovules 1994 during embryo development. A. Cones from Boise Cascade BC-1. B. Cones from UC-5-1036. C. Cones from UC-7-1051.





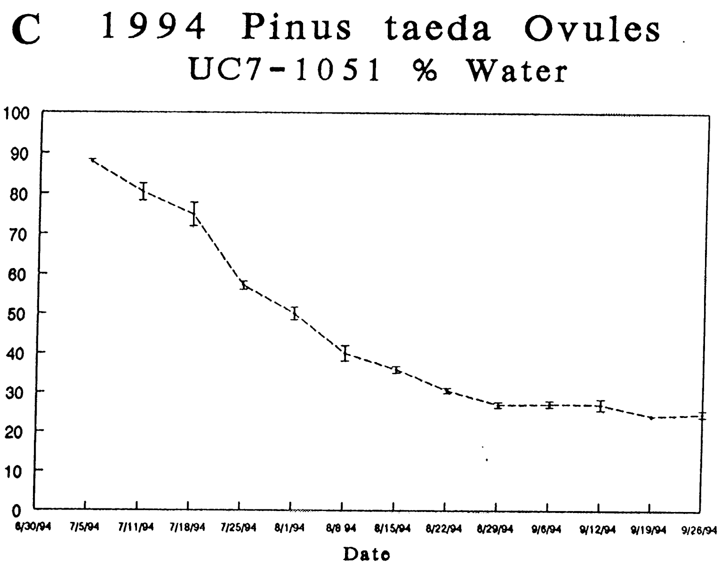
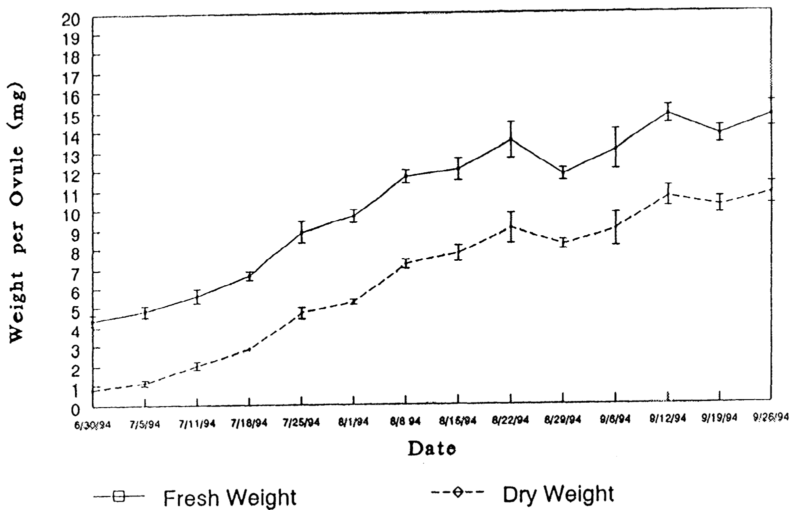
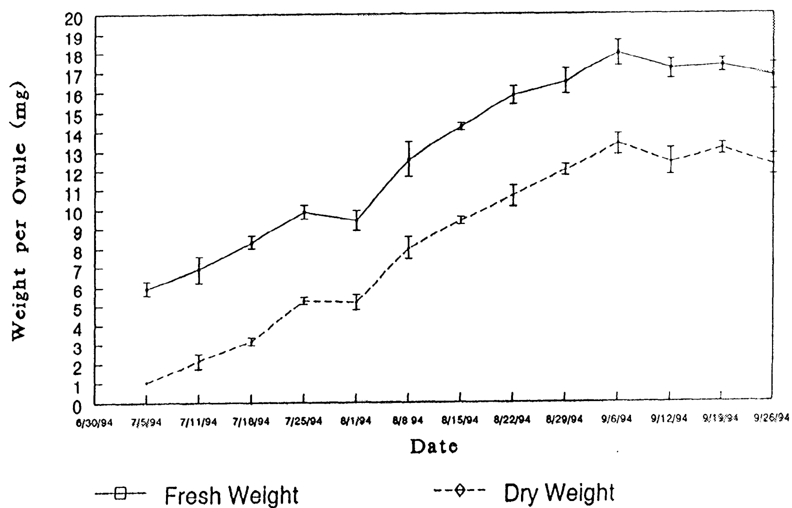


Figure 9. *Pinus taeda* whole ovule fresh and dry weight during 1994 embryo development. A. Cones from Boise Cascade BC-1. B. Cones from UC-5-1036. C. Cones from UC-7-1051.





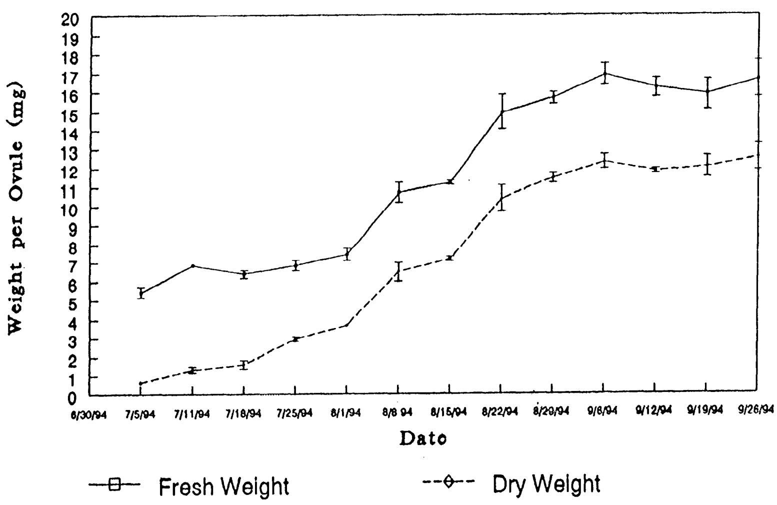


Figure 10. *Pinus taeda* whole ovule osmolality and embryo stage during 1994 embryo development. Note that as ovule osmolality begins to drop embryo development coincides with stages 4-5. A. Cones from Boise Cascade BC-1. B. Cones from UC-5-1036. C. Cones from UC-7-1051.

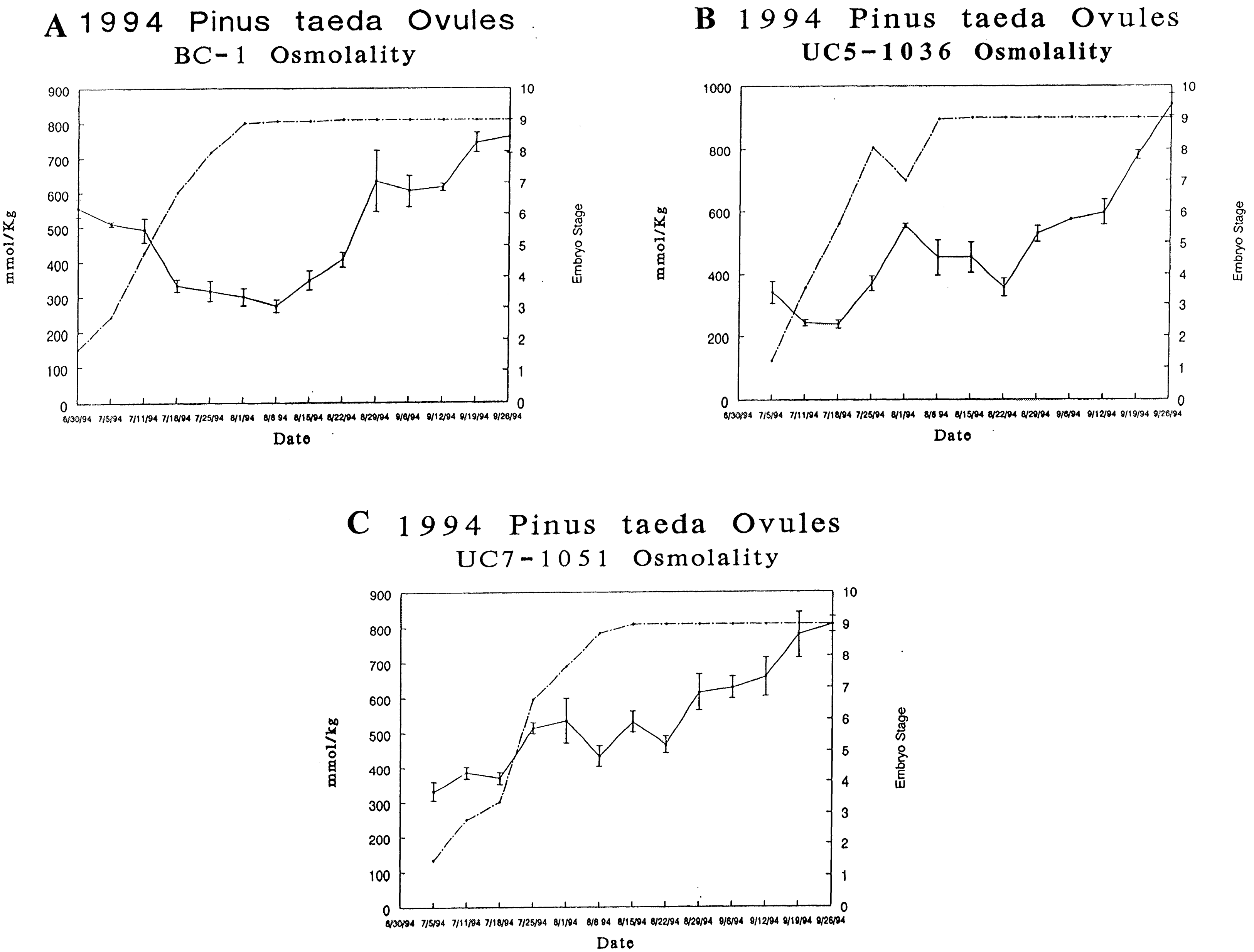
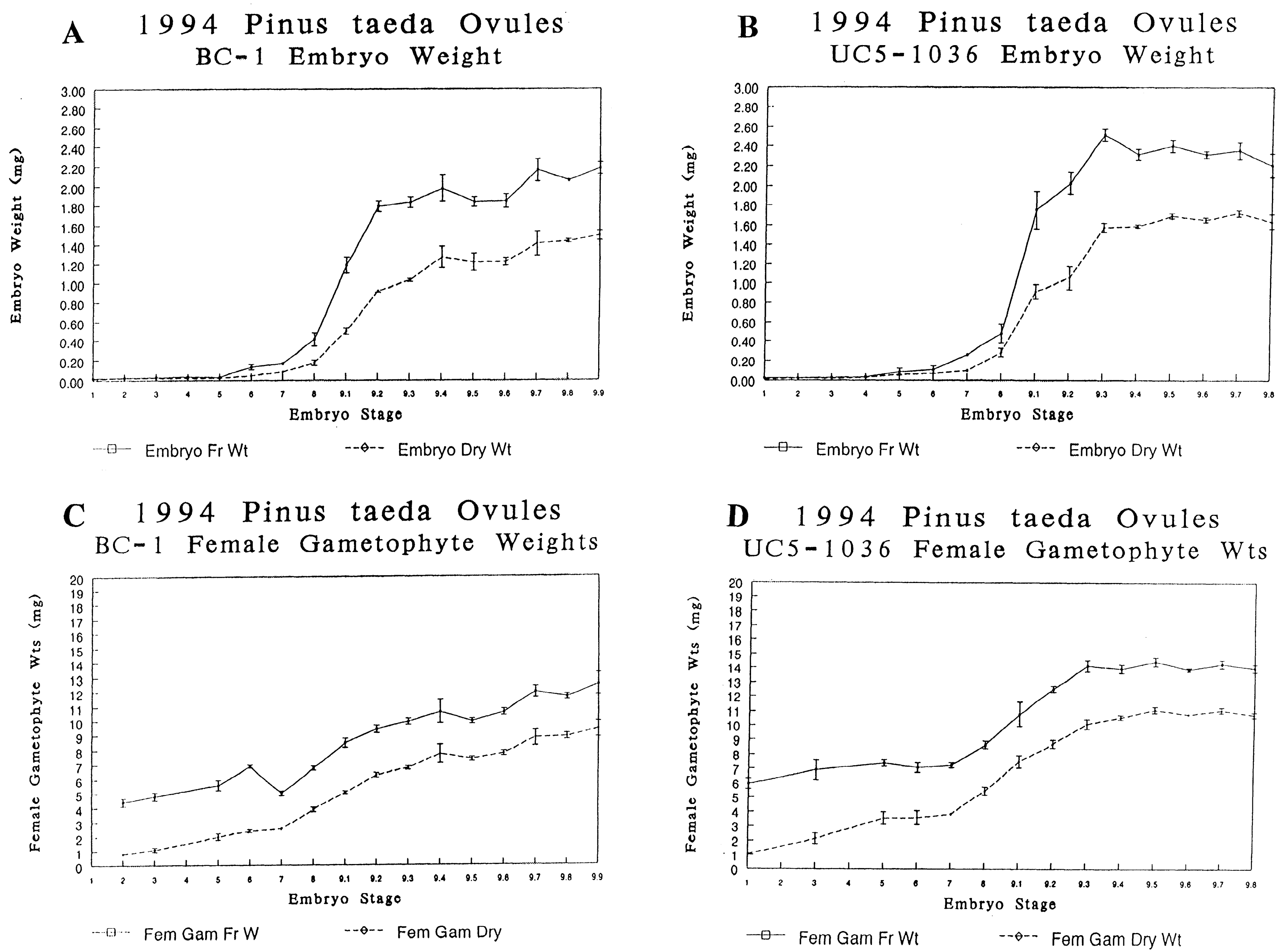


Figure 11. *Pinus taeda* embryo and female gametophyte fresh and dry weights during 1994. A. Embryo fresh and dry weights for Boise Cascade BC-1 cones. B. Embryo fresh and dry weights for UC-5-1036 cones. C. Female gametophyte fresh and dry weights for Boise Cascade BC-1 cones. D. Female gametophyte fresh and dry weights for UC-5-1036 cones.



Development of a direct ELISA for Indole-3-Acetic Acid

Vincent Ciavatta
Dr. Ron Dinus
Dr. Jeff Dean

March 21-22, 1995

Development of a direct ELISA for Indole-3-Acetic Acid

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BACKGROUND

Somatic embryogenesis is a powerful tool in tissue culture. It allows for many plants possessing a desirable genotype to be rapidly produced without experiencing severe epigenetic effects. However, development of a somatic system varies from species to species and also within species, and loblolly pine has proven to be problematic in this regard.

Trial and error experimentation has typified much of the research on somatic embryogenesis. Earlier research has shown that a sequence of media with varying growth regulator composition is necessary to induce and maintain development of somatic embryos. This need for sequential exposure to different concentrations of growth regulators has led to the theory that determining conditions during zygotic embryogenesis would provide a good model for somatic embryogenesis.
Consequently, the goal of my research is to develop a direct ELISA (enzyme-linked immunosorbent assay) to measure amounts of indole-3-acetic acid (IAA) in developing loblolly pine zygotic embryos. (Dr. Kapik used a similar technique in the development and implementation of an indirect ELISA to measure abscisic acid (ABA).

The project can be broken down into three fundamental stages: synthesis of IAA-biotin, purification of IAA-biotin, and optimization of the ELISA. IAA-biotin is required in the direct ELISA in order to compete with free methyl-IAA (the monoclonal antibody binds strongest to methyl-IAA) for monoclonal antibody antigenic sites (Figure 1). The biotin moiety is required so the massive chromogenic power of streptavidin poly-horseradish peroxidase may be utilized. This increase in color producing potential will reduce the amount and cost of monoclonal antibody, and increase overall assay sensitivity. Hence IAA-biotin is crucial to development of the assay.

We have succeeded in synthesizing a novel IAA-biotin via an amide formed from the carboxyl group of IAA and a primary amine group biotin DADOO (Figure 2). (The biotin DADOO was purchased from Boehringer-Mannheim upon the suggestion of Dr. Dean of UGA.) Furthermore, separation of IAA-biotin from free IAA and other reaction constituents has been completed by reversed-phase HPLC. GC/MS will be used to confirm synthesis of IAA-biotin. IAA-biotin via HPLC has shown favorable results in recent "test" ELISA's. Currently, the focus of my work is optimization of constituents used in the ELISA. With time permitting, mock ELISA trials with known concentrations of methyl-IAA will be performed to demonstrate usefulness of the assay.

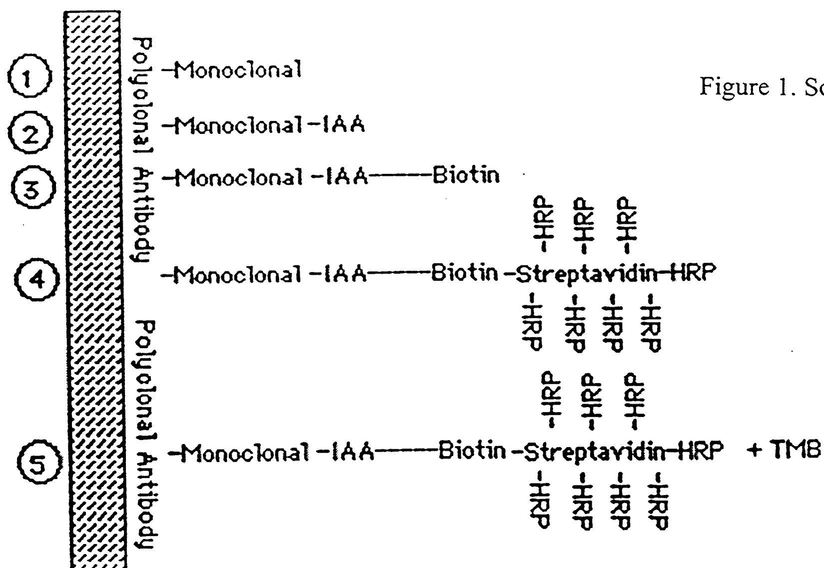


Figure 1. Schematic of direct ELISA for IAA.

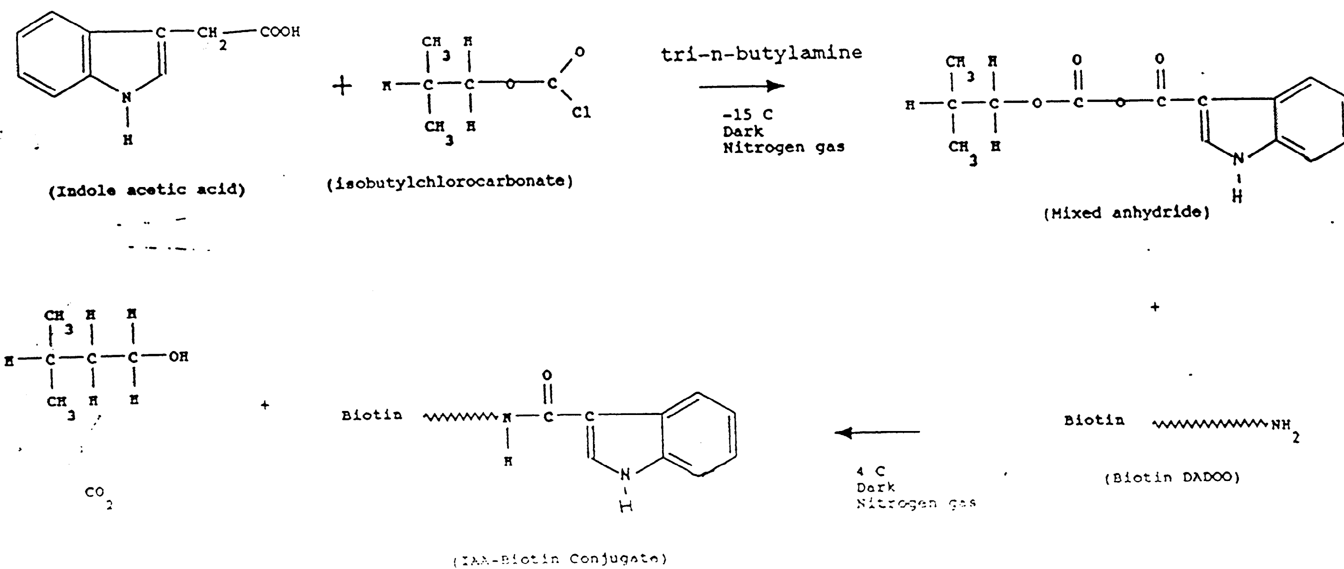


Figure 2. Synthesis of IAA-Biotin.

MOLECULAR BIOLOGY

Softwood Genetics/Molecular Biology

The Objectives of the Molecular Biology Program at IPST in the preceding 12 months have been;

1. To establish the molecular biology techniques of gene cloning, gene analysis, gene transfer and transgenic plantlet regeneration necessary to compete as a world class plant research program.

2. To initiate, expand and develop research programs of importance to the Pulp and Paper Industry.

3. To seek external Research Funding in an effort to expand and develop Biological Research at IPST

1. ESTABLISHING MOLECULAR BIOLOGY TECHNIQUES

The following techniques were established and are now operational in our laboratory. They will be referred to during the course of this report and during oral presentation.

Regeneration of Poplar plantlets from Tissue Culture
Transformation of Poplar and regeneration of transgenic Poplar plantlets (genes: GUS, *iaa*)
Regeneration of Tobacco plantlets from Tissue Culture
Transformation of Tobacco and regeneration of transgenic Tobacco plantlets (genes: GUS)
Regeneration of Arabidopsis plantlets from Tissue Culture
Transformation of Arabidopsis and regeneration of transgenic Arabidopsis plantlets (genes: GUS)
DNA Cloning into cloning vectors, transformation vectors and expression vectors
Polymerase Chain Reaction (as a means of confirming transformation results and of isolation gene fragment of interest)
Reverse Transcription-PCR - as a means of quantitating gene expression and of identifying transcripts specifically.
Northern Analysis (Radioactive)
Southern Analysis (Radioactive)

These techniques will be supplemented as over the course of the year as new techniques are introduced to answer specific problems.

2. TO INITIATE, EXPAND AND DEVELOP RESEARCH PROGRAMS OF IMPORTANCE TO THE PULP AND PAPER INDUSTRY.

Summary Report on Molecular Biology Research

- Σ *Manipulation of Lignin Synthesis*
- Σ *Drought Stress Protection*
- Σ *Regulation of Proteinase Inhibitor Gene Expression*
- Σ *Gene Expression and Somatic Embryogenesis*

<u>Manipulation of Lignin Synthesis using Gene Technologies</u>

"Antisense Technology", the introduction of reverse-orientation genes whose RNA serves to attenuate expression of target genes, is being used, increasingly, in agricultural research. This has been demonstrated most famously in the FlavrSavr tomato where expression of a ripening gene is turned down, resulting in slow ripening and a longer shelf life for the fruit

The principle of the technique is equally applicable to trees and to any trait of interest or importance. Work in our laboratory, on the responses of Loblolly Pine to drought stress revealed that increased lignin synthesis was a long-term response. A cDNA clone was isolated which showed strong similarity to a parsley gene involved in lignin synthesis. Intriguingly, this pine cDNA clone, LP1, was dis-similar to clones from Aspen and Alfalfa isolated by other laboratories. We are using this clone in an attempt to reduce lignin synthesis in model plants and ultimately trees of commercial interest.

In certain cases "sense" overexpression can have a repressing effect on endogenous gene expression though more often increased protein synthesis is the consequence of acquisition of a transgene. We have now cloned a "sense" orientation of the OMT cDNA into an Agrobacterial vector and transferred this into Tobacco, a model plant, to assess its effects. Transformants are being selected and will be screened in the next months.

Two MS Students, **Tom Kraker** and **Brian Klunk** have joined this project. They are engaged in projects, respectively, to isolate stem-specific genes and to isolate genomic clones of the OMT. In addition to furnishing information on the way this gene is controlled this work will allow a more controllable expression of antisense cDNA which we believe will allow lignin reduction to be effected without compromising the plants defense capabilities. RNA is being isolated from needles, stems and roots and Differential Display, a techniques which gives a "snapshot" of gene expression will be used to view mRNA species (gene products) which are present in the stem but not in needle or root. Chromosomal DNA is being isolated and will be used to construct a genomic library from which chromosomal fragments which contain the OMT cDNA will be isolated.

<u>Detailed Report on the Molecular Biology Research</u>

Manipulation of Lignin Biosynthesis Pathway by Overexpression As mentioned previously, we have cloned a putative methyltransferase (pLP1). This methyltransferase has nearly 70% similarity with parsely caffeoyl CoA O-methyltransferase. In parsely the enzyme converts the caffeoyl CoA to fruloyl CoA, an very important precussor on the lignin biosynthsis pathway.

Besides its role on lignin biosynthsis, feruloyl CoA has the other critical role in cell wall cross-linking. It can be the bridge between lignin molecules or between lignin and sugar molecules. Recent work from a variety of labs has shown that cell wall cross-linking plays a role in stress protection. We wish to determine the effect of OMT overproduction on lignin quantity, quality and plant growth under different conditions.

In order to subclone the pLP1 fragment into a plant transformation vector in the correct orientation we developed the following strategy.The full length cDNA of LP1 is about 1.1kb and is cloned into the SK+ Bluescript plasmid. The pLP1 was digested with XhoI, to release the cDNA fragment, followed by a blunt-ending with T4 DNA polymerase. The linear, blunt ended molecule was then digested with XbaI which has a single sight near the 5' end of the molecule. The resulting LP1 fragment is shown in Figure 1.

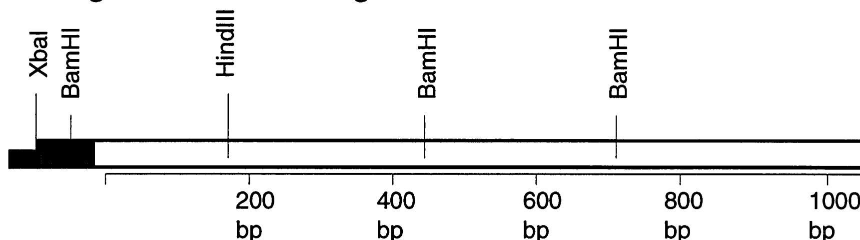


Figure 1. LP1 with modified ends, one end is XbaI compatible and the other end is blunt.

The binary vector is PBI121 (Clontech, CA). The vector was opened with Sst I and blunt ended subsequently. The linear plasmid was then digested with Xba I, resulting a vector as displayed in Fig.2.

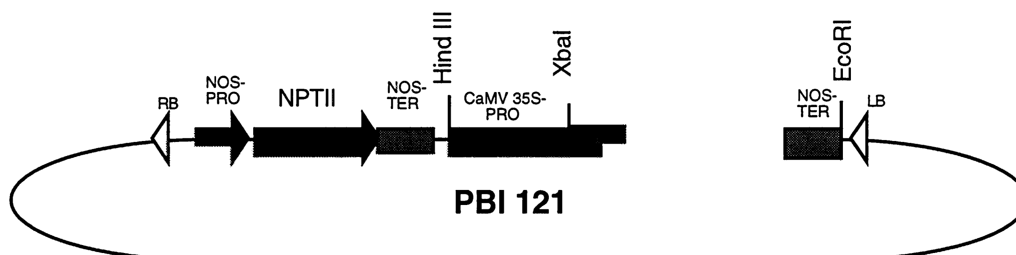
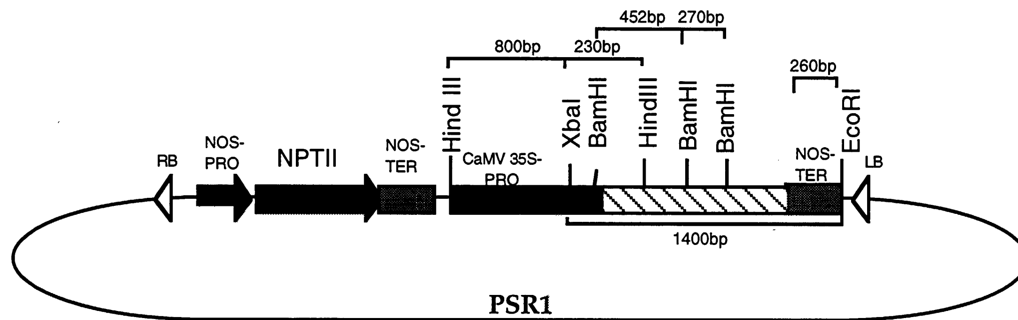


Figure 2. PBI121 was cut and modified to fit LP1 fragment.

After ligating the LP1 fragment in Fig.1 and the vector in Fig.2. The engineerred plasmid contains LP1 in a sense orientation driven by CaMV 35S promoter and the NPTII gene controlled by NOS promoter (Fig.3).



The cloning was checked by four restriction enzymes. As predicted Bam HI restriction gave two bands of the right sizes, HindIII resulted a single band about 1 kb, and EcoRI and XbaI double digestion released a fragment about 1.4kb (Fig.4).

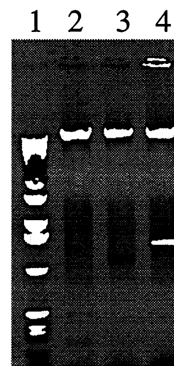


Figure 4. Restriction digestion to check the engineered plasmid. Lane 1: DNA size ladder; lane 2 and 3: pSR1 is digested by BamHI and HindIII respectively; lane 4: double digestion by XbaI and EcoRI.

The engineered plasmid, pSR1 (for Stress Resistant) was delivered into *Agrobecterium* strain LBA4404 by freeze-thaw method. Again the transfection is checked by plasmid isolation from Agrobacteria and restriction digestion.

For tobacco transformation, leaf sections were infected with the Agrobacteria with a concentration of 10^{10}. The regenerated shoots were transferred to kanamycin (150mg/L) and carbenecillin (500mg/L) containing medium for rooting. Selection and screening are in process.

Genomic Clones of OMT In order to have a better understanding on the regulation of OMT it is important to have genomic clone(s) which contains the controlling elements. This project has been started recently. The first step is to isolate high quality genomic DNA. The criteria are integrity and the digestability by restriction enzymes. So far we have successfully isolated genomic DNA from loblolly pine clone s6pt2xs6pt3. Gel picture indicates our genomic DNA is larger than 50kb (Fig.5A), and is digestable by tested restriction enzymes such as BamHI, HindIII, EcoRI, MboI, etc. (Fig.5B)

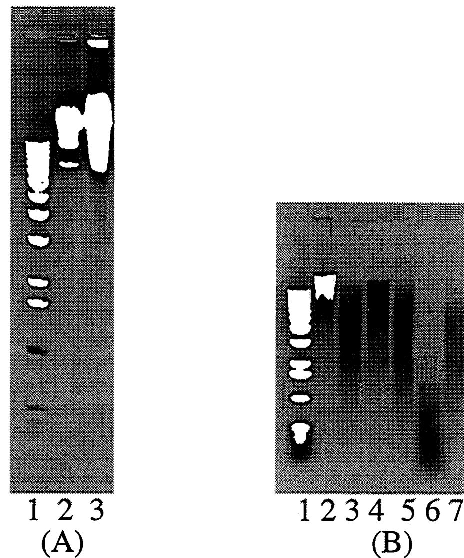


Figure 5. A) Lane 1: DNA kb ladder up to 12kb; lane 2: mass molecular weight marker 8-50kb; lane 3: undigested pine genomic DNA. B) lane 1: kb DNA ladder; lane 2: undigested DNA; lane 3: restriction with EcoRI; lane 4: restriction with HindIII; lane 5: restriction with BamHI; lane 6: restriction with MboI; lane7: double digestion with BamHI and EcoRI.

Our next step is to construct the genomic library. We are going to use the lambda BlueStar system (Novagen). This system has many advantages including high efficiency of cloning DNA fragments of 7-20 kb in size and particularly a one step excising of plasmid directly from the lambda vector. At present time we are in the process of fractioning genomic DNA fragments.

Drought Stress Protection

Site specificity is problem which confronts growers when they seek to establish a stand of high-value trees in a new location. Environmental stresses such as salt and drought stress have been suggested as possible explanations for the difficulties in cultivating clones. We are expanding a project exploring gene expression in plant subjected to drought stress to learn more about the molecular response of trees to water deficit. As the physical and biochemical perturbations caused by environmental and biological insult are often overlapping so are plant responses to them. We believe our work will shed light on a variety of stresses which are encountered by trees during growth.

Hormonal Induction of Stress Genes. (Work carried out by Dr. Shujun Chang in collaboration with Dr. Jerry Pullman). Environmentally stressed trees activate previously quiescent genes which encode defense and repair proteins. We are investigating the hormonal signal which may mediate these stress cues. Loblolly Pine cells have been grown in culture media supplemented with a) 0.15M NaCl, b) 0.3M Maltose, c) 10μM Abscisic Acid (ABA), d) 10μM Methyl Jasmonate e) no addition. Cells were harvested at 1, 4, 8, and 24 hours after supplement was

added. RNA has been extracted from these cells and expression of a number of Loblolly Pine stress-inducible genes is being assayed.

Regulation of Proteinase Inhibitor (PI) Gene Expression. (Work carried out by Ph.D. student Debbie Villalon). Pathogens such as fungus and beetles cause massive destruction of Forest Lands. Plant defenses to such pathogens include the production of Defense/Repair proteins which inhibit pathogen feeding or directly attack the invader. The genes for plant defense proteins, when cloned, overexpressed and transferred back into plant cells, can confer upon transgenic plants, enhanced tolerance to pathogens and other environmental stresses. A project is being conducted by Ph.D. student Debbie Villalon, to analyze the function of a Proteinase Inhibitor and to examine the regulation of the gene which encodes it.

Several cDNA clone of a PI gene were isolated from the woody desert shrub *Atriplex canescens* in a previous study (see Figure 6). The PI gene was shown to be induced by water deficit as well as a number of other stresses. To evaluate the effect of this defense gene, we used a model plant, *Arabidopsis thaliana*, where experiments could be conducted swiftly. The rapid growth of this plant, its molecular similarity to commercial tree species, combined with the genetics and molecular biology research which has generated knowledge, mutants and DNA clones, make *Arabidopsis* an excellent model for research.

The tools necessary for such reseach were established. Regeneration techniques for producing plantlets from culture were developed. (See Figure 7). Gene transfer, using an easily assayable GUS gene under a constitutive promoter, was carried out using *Agrobacterium tumefaciens* (See Figure 7). The blue areas of tissue indicate that the plants contain and express the GUS gene. Techniques of regeneration and gene transfer are now working routinely in the laboratory.

We wished to "overexpress" the PI gene as a means of conferring enhanced protection on plants as has been demonstrated in other plants. First we wished to evaluate the efficacy of the protein against a range of proteinases. To do this a PCR fragment which encodes the protein was generated and cloned into the PinPoint Xa protein purification system (Promega, Madison, WI). This plasmid vector allows large amounts of a biotinylated fusion protein to be produced in *E.coli*. The biotinylated fusion protein can be purified by column chromatograph by binding to avidin resin and subsequent release under nondenaturing conditions by competitive binding of biotin. The purified protein can be cleaved specifically betwen the biotinyated tagged protein and the target protein therby releasing the proteinase inhibitor-I.

An assay for enzyme inhibition is being developed with specifictiy to trypsin and chymotrypsin. The radial inhibitor diffusion assay involves the application of protein to an enzyme-containing agar film and a staining technique based on chromogenic substrate *N*-acetyl-DL-phenylalanine-β-naphthyl ester (APNE). Enzyme inhibition is visualized as colorless zones on a pink background after films are stained. Measurements of the diameters of the colorless inhibition zones are proportinal to the amount of inhibitor applies to the gels. The function of this protein is now being assayed in vitro using a Proteinase Inhibitor assay.

Control of Stress Gene Expression. I. Transcriptional. (Work carried out by Ph.D. student Debbie Villalon). Stress genes are "activated" by appropriate environmental signals. Appreciating

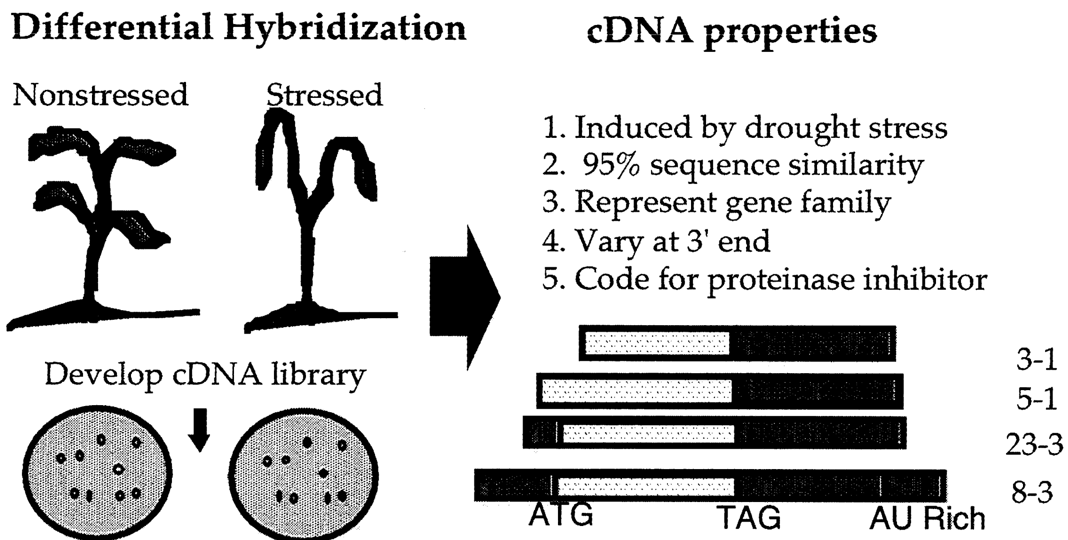


Figure 6.

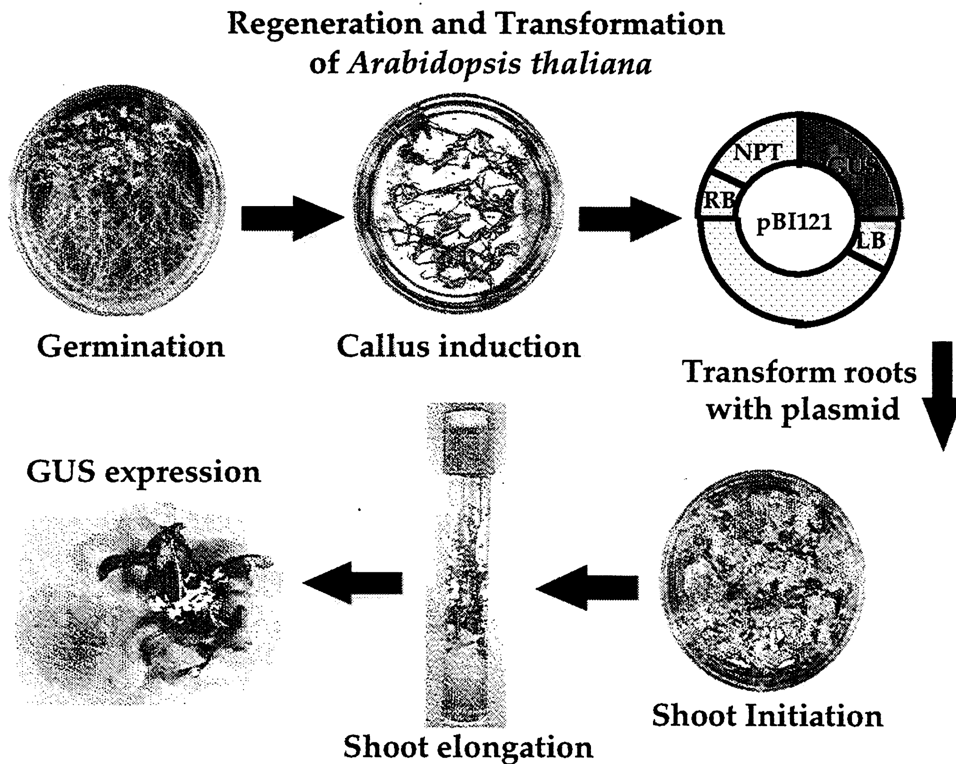


Figure 7.

the molecular mechanisms which effect this induction will allow us to evaluate our approaches to tree protection through molecular biology and design promoters which better suit the needs of the grower.

Two genomic copies of the PI gene have been isolated from a genomic library by Debbie Villalon. These were named pG12-95 and pG18-1 and their features are diagramed in Figure 8. The genes comprise of two exons separated by one inton. The predicted amino acid sequence is virtually identical to those of the cDNA clones though sequence differences suggest that pG12-95 and pG18-1 are additional members of this gene family. Each genomic clone contains one intron of 931 and 422 bp respectively. The putative promoter elements of these genes have been analysed and several sequence motifs for established Transcription Factors have been recognized The sequence elements (which include basal elements such as CAAT boxes and TATA boxes) are found in many genes which respond to wounding and environmental stress. Some, such as the ABA Response Element (ABRE) are involved in mediating the hormonal induction of gene expression. In addition the genes possess unusual sequences in their promoter regions which we wish to analyze further. Promoter fragments are being isolated using PCR and cloned into appropriate vectors for assay. By removing and/or modifying sequences we will be able to determine how they function in transgenic plants.

Control of Stress Gene Expression. II. Post-Transcriptional. While control of the production of mRNA is the most frequently encountered form of controlling the amount of protein produced, transgenic work and more careful evaluation of gene expression has shown that the translation of mRNA is exquisitely regulated. The implication of this previously underestimated phenomenon is that simply transferring a gene into a plant and insuring that mRNA is produced from it, does not guarantee that the desired protein will be synthesized in the amount or the location which is desired. To examine post-transcriptional regulation of gene expression we have focused on two genes, previously cloned by us, which exhibit features which strongly indicate that extensive regulation of translation and/or message stability is occurring.

Proteinase Inhibitor-1 Gene Expression

The PI-1 gene discussed in the last section appears to be expressed as two size classes of mRNA, 0.55kb and 0.7kb. Preliminary evidence from the Northern analysis suggest that the longer transcript appears at more severe levels of desiccation. We have isolated and sequenced several cDNA clones which may correspond to the size classes observed. The clones represent members of a multigene family and encode essentially the same protein and differ principally in the length of the 3'untranslated region (3'UTR) of their mRNA. Size differences may imply that the length of their polyA tail is different. The length of the polyA tail is a feature which influences the stability of an mRNA and the efficiency with which it is translated.

Northern Analysis does not distinguish between molecules which have close sequence similarity therefore we are attempting to distinguish between the mRNAs and to determine when they are expressed using the technique of Reverse Transcription-PCR (RT-PCR). Oligonucleotides were designed which were specific for each of the cDNA clones. These oligonucleotides should generate fragments of characteristic size during RT-PRC. To demonstrate their specificity each set of oligonucleotides was used to amplify its specific sequence from a mixture of all the cDNA

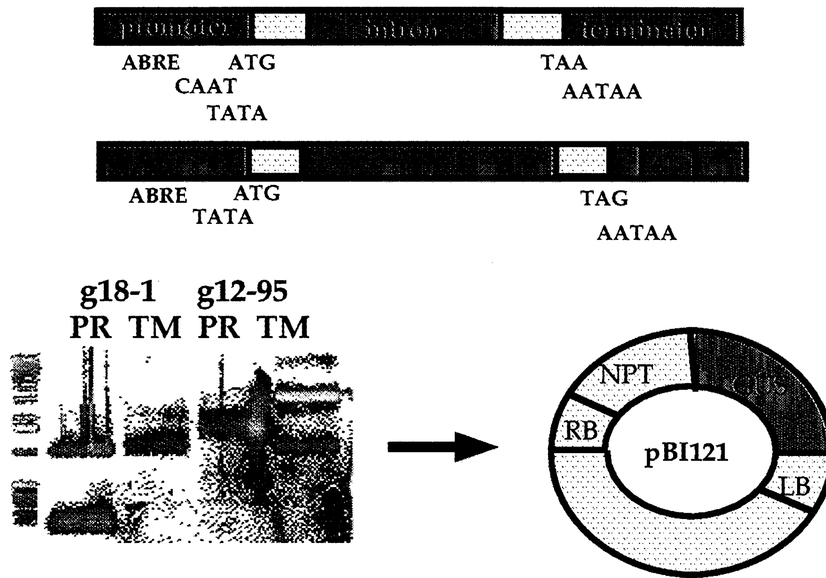


Figure 8.

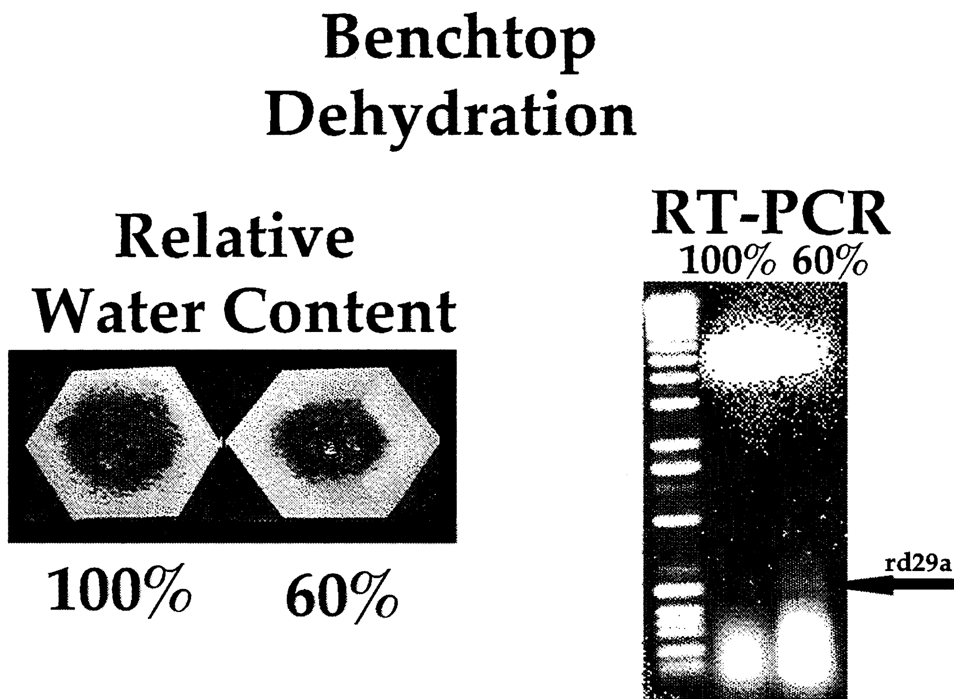


Figure 9.

clones. Through repeated experimentation we were able to identify conditions which permited the amplification of specific sequences from among a mixture of plasmids. We will now use these techniques to analyse the expression of particular PI genes.

To examine drought in Arabidopsis we had to establish dehydration protocols for this plant. The method most commonly used is that of air drying plants on the bench and assessing dry weight before determning gene expression. To demonstrate that plants were being dehydrated and that the techniques of RT-PCR was sensitive enough to detect changes in gene expression we carried out control experiments to detect expression of a desiccation-inducible gene which had been isolated in another laboratory. Oligonucleotide primers were designed to the rd29, a dessication-inducible gene from Arabidopsis, and RT-PCR was conducted in control and desiccated plants. Figure 9 shows that a band is generated (thus RNA is present) only in desiccated plants. Having established conditions for specific sequence amplification and for drought-gene induction we are now in a position to assay the induction of particular copies and classes of PI-1 gene in Arabidopsis.

Analysis of a Chitinase homolog - the pLP6 Gene Family .

A 1.5 kb cDNA clone, pLP6, was isolated by us from loblolly pinea and was shown to be homologous to a number of class I chitinases from bean, tobacco and poplar. This gene is down-regulated by water deficit stress (Fig.10). One of the very unusal features of this cDNA is the 721 bp 5' untranslated region (5'UTR), which comprises almost half the length of the cDNA (hence the corresponding mRNA). In this 5'UTR, there are a number of stem loop-forming inverted repeats (Fig.11). These repeats are very stable based on the predicted secondary structure energy and are very similar to stem loops found in ferritin genes. In ferritin, these stem-loops interact with proteins, an interaction which controls mRNA translation. In plants the CaMV 35S transcript has a 600bp 5'UTR which possesses many stem-loops whose function has not yet been determined. The length of the 5'UTR and the presence of several inverted repeats raise the possibility that interaction with proteins may regulate LP6 mRNA translation.

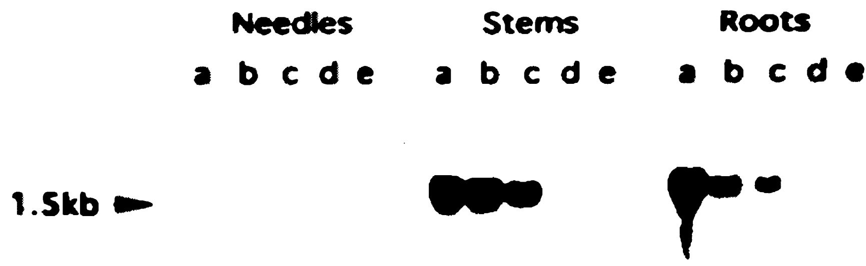


Figure 10. LP6 is down regulated by drought stress. Lanes a - e represent water potential of -0.35, -0.8, -1.35, -1.8, and -2.3 Mpa respectively.

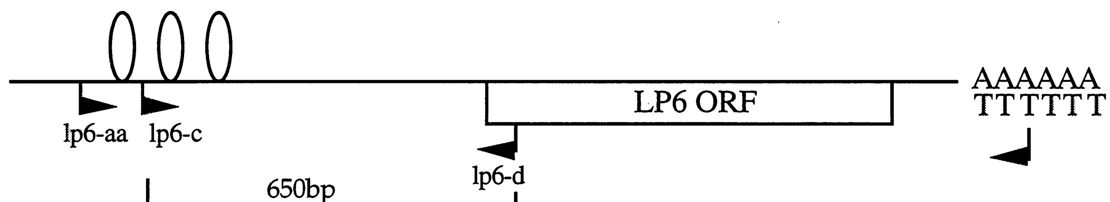


Figure 11. Simplified diagram of LP6 structure. Lp6-aa, c, and d are different sequence-specific primers.

Since pLP6 possesses such and unusual structure we wished to establish whether a) the clone was really derived from a single mRNA or whether it was the result of artefactual joining of two mRNA (or cDNA) and b) whether there were additional copies of this gene or related genes in the pine genome. To answer these questions we used RT-PCR, a powerful technique combining the synthesis of cDNA from mRNA by reverse transcriptase (RT) and polymerase chain reaction (PCR). Our hypothesis is that if the 5'UTR and ORF belong to different genes and are on different mRNA molecules, then sequence-specific primers lp6-c (in 5'UTR) and lp6-d (in ORF region) would not be able to amplify the 650 bp region flanked by these primers (Fig. 11). If on the same mRNA amplification will occur.

We used 5µg total RNA from the stem of a well irrigated loblolly pine seedling for RT reaction. One tenth of the RT products were used for the subsequent PCR with lp6-c and lp6-d as primers. To our surprise we amplified three sharp bands, a strong band about 800 bp, a weak band about the right size (650bp), and a strong but smaller band around 400 bp (Fig.12A). Is the middle band the right one? The weak band was cut off the gel with a clean razor blade and the DNA was isolated from the gel. The purified middle band DNA was reamplified by the same primers. Meanwhile the pLP6 plasmid DNA was amplified by lp6-c and lp6-d as a positive control. Fragments of identical size were obtained from both PCR reactions (Fig. 12B). This experiment suggests strongly, that the 5'UTR and ORF do belong to one gene and additionally the result implies that LP6 is a member of gene family. The larger and the smaller bands are even more aboundant than LP6. The gene family idea is also supported by a different RT-PCR reaction where lp6-aa and oligod(T)n were used as primers (Fig. 12C). Multiple sharp bands

were amplified (Fig 12C), indicating the presence of many genes with strong homology to the region defined by the primers.

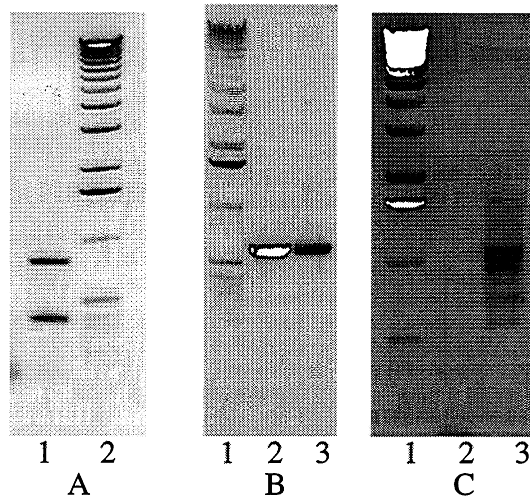


Figure 12. A) RT-PCR with lp6-c and d as primers (lane 1). B) Reamplified middle band (lane 3) has the same size as the PCR using LP6 plasmid as a control (lane 2). C) RT-PCR with lp6-aa and oligod(T)n as primers (lane 3).

We will now confirm the identity of the small fragment by DNA sequencing. The presence of additional bands raises intriguing possibilities; is the 5'UTR a feature of a number of genes thus allowing for their co-ordinate regulation.? Are these genes closely related to pLP6 or quite dissimilar, simply responding to the same biochemical cues? The PCR fragments can now be cloned, sequenced and subcloned into plant transformation vectors for functional assay. We will now be able to ask, do these sequences reduce or enhance the expression of a reporter gene? Does this behaviour vary with growth conditions? Doee it respond to hormone aplication?

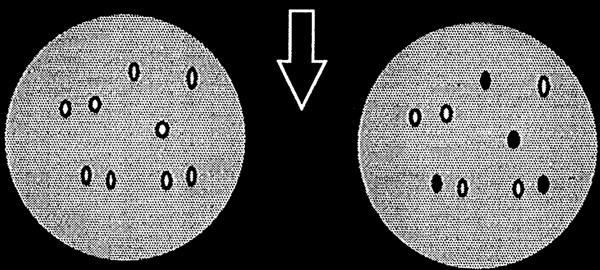
```
1   S D Q S - - S S Y - - - - - - - - D D D E Y S K P C C D L C M C T R S M P P Q C  Soybean
1   L S M R F F A T F F L A - M L V V A T K M G P M R I A E A - - - - - - - - - R H C  Potato
1   - - M R P I A V F F L V F L L A L T T E E V G P R V A E G F L - - - G I G K K C  Atriplex

31  S C E D I R L N S - C - - H S D C K S - C M C T R S Q P G Q C R C L D T N D T N  Soybean
32  E S L S H R F K G P C T R N S N C A S V C E T E R F S G G N C H G F R R R C - -  Potato
36  S I P S K T K K G P C F S D T N C D S I C R A E R M R A G I C H G V R R R C - -  Atriplex

67  D F C Y K P C K S R D D  Soybean
70  - F C T K P C            Potato
74  - L C C R                Atriplex
```

Figure 3. Comparison of amino acid sequences of proteinase inhibitor CII (Bowman Birk) of soybean, potato clone p322 (W.J. Steikema et al., 1988), and predicted polypeptide of *Atriplex canascens* clone 23-3.

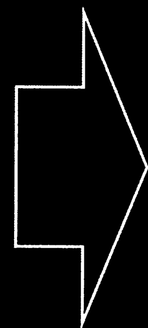
Differemtial Hybridization

Nonstressed Stressed

Develop cDNA library

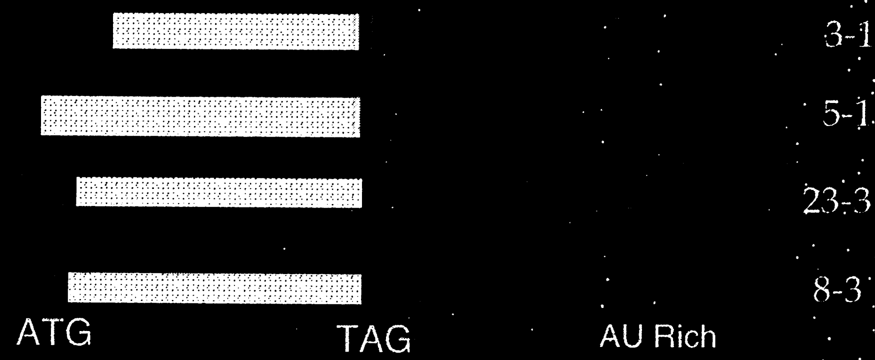


Select stress
induced clones



cDNA properties

1. Induced by drought stress
2. 95% sequence similarity
3. Represent gene family
4. Vary at 3' end
5. Code for proteinase inhibitor



HARDWOODS

MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS

Status Report for
Project F011

Ronald J. Dinus
Camille J. Stephens
Shujun Chang

March 21-22, 1995

Institute of Paper Science and Technology
500 10th Street, N.W.
Atlanta, GA 30318
(404) 853-9500

Technical Program Review

Project Title:	**MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS**
Project Code:	HRDWD
Project Number:	F011
PAC:	Forest Biology
Division:	Chemical and Biological Sciences
Project Staff:	R. Dinus, C. Stephens, J. Cairney
FY 94-95 Budget:	$133,000

PROGRAM OBJECTIVE:

Develop reliable cell and tissue culture systems for mass clonal propagation of genetically improved and/or engineered hardwoods.

SUMMARY OF RESULTS:

This project seeks to build technologies for transferring genes for increased resistance to stress into commercially important hardwoods, and for effective propagation, testing, and release of genetically modified trees. Examples of stress resistance genes include herbicide and drought tolerance.

Greenhouse Clone Bank: Plants derived from culture are being multiplied to replace older, less vigorous greenhouse ramets. New clones, several of which are sexually sterile, have been added to extend research to commercially valuable clones. Working with sterile clones should facilitate obtaining permits for long term field tests of transformed plants.

Refining the Transformation Protocol: Our recently published technique was used to move three elite clones into culture for future transformation trials. A lethal dose assay with the selective antibiotic, kanamycin, showed that a concentration of 400 mg/L eliminates nontransformed explants from cultures of greenhouse internodes of model clone K417. Results from two experiments showed that internode explants obtained from K417 cultures are more responsive to shoot induction protocols than greenhouse internodes. In the first trial, 95% of the explants produced shoots; yielding 13 shoots per explant over a 181 day culture life. Internodes from culture should therefore b e viable vehicle for transformation, especially for clones not easily manipulated via the leaf section system used earlier. Taken together, results from these several trials pave the way for transformation experiments with a greater variety of explants and clones.

Earlier student research on transformation was plagued by lingering contamination with Agrobacterium tumefaciens (At), the bacteria used to effect transformation. Any loss of explants to overgrowth At reduces the probability of transformation. The antibiotic, carbenicillin, normally used to rid cultures of At may be too sensitive to light and temperature for effective use during the lengthy culture periods required for cottonwood. Evidence also exists that an enzyme secreted by At may degrade carbenicillin. To circumvent such problems, replicated lethal dose assays tested sensitivity of At and model cottonwood clone, C175, to cefotaxime, an antibiotic more stable and longer lasting than carbenicillin. Cefotaxime concentrations of 250 and 500 mg/L stopped growth of At without interfering with callus, primordia, or shoot formation on C175 leaf sections. Accordingly, cefotaxime is being used in place of or in tandem with carbenicillin.

Transformation Trials: Seven transformation experiments have been established since start of the fiscal year; two with the iaaM gene for enhanced auxin synthesis and five with a benign marker gene, the GUS Reporter Gene. Also evaluated in these trials were various measure to heighten probability of transformation; e.g., varying concentrations of and lengths of exposure to At. Results from the four trials that have been completed or are nearing completion are summarized below.

In all experiments, control leaf sections not exposed to At or kanamycin, the antibiotic used to select for transformants, formed callus, primordia, and shoots in accordance with expectation, clearly demonstrating that leaf sections used in the tests were healthy and responsive. Leaf sections not exposed to At but cultured on Kanamycin were all killed, confirming that 150 mg/L of this antibiotic is sufficient to eliminate nontransformed tissues.

Leaf sections exposed to At but not kanamycin formed callus, primordia, and shoots later than control explants, but levels of production eventually approached those of controls. Heightened At concentrations and lengthened exposure times, however, slightly reduced shoot yields. Thus, exposure to At and the manipulations associated with the transformation protocol do not disrupt regeneration from leaf sections.

Shoots from such cultures are putative transformants, and have been rooted for growth on shoot growth medium containing kanamycin; ie., selection of transformants is being done at the shoot rather than tissue level. Nearly 100 such shoots are being grown in the presence of 50 mg/L kanamycin; those surviving are being or have been transferred to medium containing higher kanamycin doses. Any surviving this treatment will be screened for the presence of iaaM and GUS genes via biochemical and molecular assays. A limited number of shoots surviving the initial (lower) exposure have been assayed, but no evidence of transformation has been found to date.

In all trials and as expected, most leaf sections exposed to AT and kanamycin eventually were killed, indicating that their

transformation had not been effected. Small but significant numbers, however, have remained alive, and have formed or are forming callus and/or primordia. No shoots have been harvested to date, but promising cultures are Being stimulated to foster development. Samples have been collected from the more vigorous cultures for biochemical and molecular confirmation of transformation. Although assays are still in progress, four cultures have given positive tests for the GUS gene, and presence of the iaaM gene has been confirmed in five cultures, clear confirmation of successful transformation.

Other results suggest that higher concentrations of and longer exposure to At may raise frequency of transformation without greatly diminishing regeneration efficiency, indicate that residual At contamination can be reduced by frequent rinsing in antibiotic solutions, confirm that cefotaxime aids in holding At contamination at lower levels than carbenicillin, and suggest that holding leaf sections on nonselective medium for a short period of time after exposure to At and before culture on selective medium may improve transformation efficiency.

External funding ($27,940) is being used to support efforts during the current fiscal year. Renewal for slightly higher funding ($58,790) has been sought and is expected.

INTRODUCTION:

Populus species and hybrids are among the fastest growing and most commercially important hardwoods in the world. Eastern cottonwood (P. deltoides) is especially noted for rapid growth and desirable fiber properties. Significant genetic improvement has been obtained via classical selection and breeding; however, more rapid and specific improvements can be achieved by more direct manipulation of genes.

Genetic transformation via Agrobacterium tumefaciens (At) is one approach being evaluated at IPST. Traits of interest include enhanced auxin synthesis, which has potential for altering fiber numbers and/or dimensions (Dinus and Stephens 1992). Increasing fiber numbers could raise volume productivity. Changing fiber dimensions, e.g., longer fibers with thinner walls, could lead to improved or new paper products. Also, the ability to control auxin status at will in trees or tissue cultures will foster research on mechanisms underlying tree growth and development, including those affecting xylem cell division, differentiation, and elongation.

Klee et al. (1987) transformed Petunia hybrida VR with a gene (iaaM)for enhanced auxin synthesis, and observed numerous changes in the morphology and anatomy of transformed plants. Leaves were much smaller, thinner, and curlier than those of normal, wild-type petunia plants. Adventitious roots formed on leaves and stems of many plants. In addition, transformants produced twice the normal number of secondary xylem and phloem cells, had elongated and woody stems, and exhibited extreme apical dominance. These outcomes were

attributed to overproduction of auxin, as transformed plants were found to have 10-fold more auxin than wild-types. Despite such large changes, transformants were fertile, and selfed offspring had the same phenotypes as parents.

IPST attempts at genetic transformation began with transfer of the iaaM gene into eastern cottonwood clone C175, a genotype noted for ease of manipulation in culture. Construct pMON518, containing the iaaM gene coupled to a 19S constitutive promoter, was obtained from Monsanto Corp. The Leaf Section System developed earlier at IPST for propagating cottonwood (Dinus 1992) was modified for use in transformation with At (Shorter 1991). Such systems have been used for transformation of Populus hybrids with At (Fillatti et al. 1987, Sellmer and McCown 1989).

First efforts at IPST produced several putatively transformed calli and primordia (Dinus and Stephens 1994). All structures eventually reverted to calli, but continued to grow on selective medium. Appearance and growth resembled those of cottonwood cultures given excess auxin. These symptoms were taken as preliminary evidence of transformation. Southern blotting with the digoxigenin system confirmed presence of the iaaM gene (Dinus and Stephens 1994). Plants, however, were not recovered, presumably a result of excess auxin interfering with regeneration from the normally efficient Leaf Section System. Frequency of transformation, averaging 1% over two trials, was similar to that noted in the literature, but far too low for efficient, routine application of the protocol.

Accordingly, research was continued, with the aim of improving protocol efficiency. Objectives for Fiscal Year 1994-95 include:

* Effect transformation of a model cottonwood clone with a benign marker gene and constitutive promoter and with the iaaM gene coupled to a controllable promoter;

* Regenerate transformed plants;

* Confirm transformation of cultures/plants via biochemical and molecular assays; and

* Document effects of iaaM gene expression on early growth and external morphology of transformed cultures/plants.

Toward these ends, a variety of experiments has been initiated and/or completed; results to date are recounted below. Experiments are grouped according to individual objectives, with sections on materials and methods, results, and conclusions given within the discussion of each individual trial or series of experiments.

REFINING AND EXPANDING THE TRANSFORMATION PROTOCOL

GREENHOUSE CLONE BANK

Plants derived from cultures of five cottonwood clones, including three elite clones, were prepared for transfer to the greenhouse as part of an ongoing effort to replace older ramets. Additional clones were obtained from cooperators, James River Corp. and the poplar breeding program at the University of Washington and Washington State University. Included are several triploid hybrids known to be sexually sterile; regulatory agency permission to field test transformed plants derived from sterile materials can be obtained with ease. Collectively, these actions ensure continuing supplies of material for research, and permit extension to elite clones of known commercial value.

ESTABLISHING ELITE CLONES IN CULTURE

Our recently published technique for producing shoots from greenhouse internodes (Stephens et al.1994) was used to establish three elite clones in culture. The goal was to provide material for research on transformation with elite clones as well as with model clones, ie., those easily manipulated in culture.

Toward this end, cultures were established with St66, one of the fastest growing clone available to southern growers; St75, a fast-growing clone with above average cellulose content; and WV96, a clone noted for high productivity in the mid-Mississippi River Basin. Percentages of explants with harvestable shoots were: St66 = 20%, St75 = 17%, and WV96 = 15%, results as good as or better than any obtained in the past. Shoots were harvested, have been rooted, and are being multiplied in culture for eventual use in transformation trials.

EVALUATING ADDITIONAL EXPLANT TYPES FOR USE IN TRANSFORMATION

Two approaches were taken to expand the type and quality of explants usable in transformation experiments. The first involved a small, informal trial comparing utility of the IPST Leaf Section System with two alternative protocols borrowed from the literature (Prakash and Thielges 1989, Stephens et al. 1994). The trial was designed to find a convenient means to transform an additional model clone, K417. The IPST protocol produced higher frequencies of callus, primordia, and shoot formation than the alternatives. As a result, attempts to transform additional clones via leaf sections will use the IPST protocol.

In the second approach, a series of experiments was undertaken to test utility of internodes collected from culture as a vehicle for transformation. Two trials were initiated with the model clone, K417, a clone that yields large numbers of harvestable shoots from greenhouse internodes (Stephens et al. 1994). We hypothesized that internodes collected from culture might work well too, and

therefore be an effective substitute for transformation with leaf sections. K417 is not as easily regenerated via leaf sections as are other clones, e.g., C175.

The first, exploratory, trial tested one of several protocols found useful with greenhouse internodes (Dinus et al. 1993): exposure to callus inducing medium (CIM) (MWPM + 0.5 mg/L 2,4-D) for four days (CIM-4) followed by culture on standard shoot elongation medium (MWPM + 1.0 mg/L zeatin) (SIM). The experiment included 22 petri plates (replications), each containing 10 internode explants. Percent of explants forming callus, primordia, and harvestable shoots as well as numbers of harvestable shoots per explant were recorded at varying intervals through 181 days in culture.

Results were most promising, with internodes from culture forming callus, primordia, and shoots faster than their counterparts from the greenhouse (Figures 1 and 2). Callus and primordia formed on 100% of the explants, and shoots had been harvested from 95% of the explants by the time the experiment was terminated. Cumulative yields of harvestable shoots averaged 13.4 per explant. Such performance, especially in terms of harvestable shoot yields and productive lifetime, suggest that internodes from culture are a viable vehicle for transformation.

Figure 1. K417, Internodes from Greenhouse. Time Course of Development Following a Four-Day Exposure to 2,4-D (CIM-4) and Culture on 0.5 mg/L Zeatin (RP745, 1991)

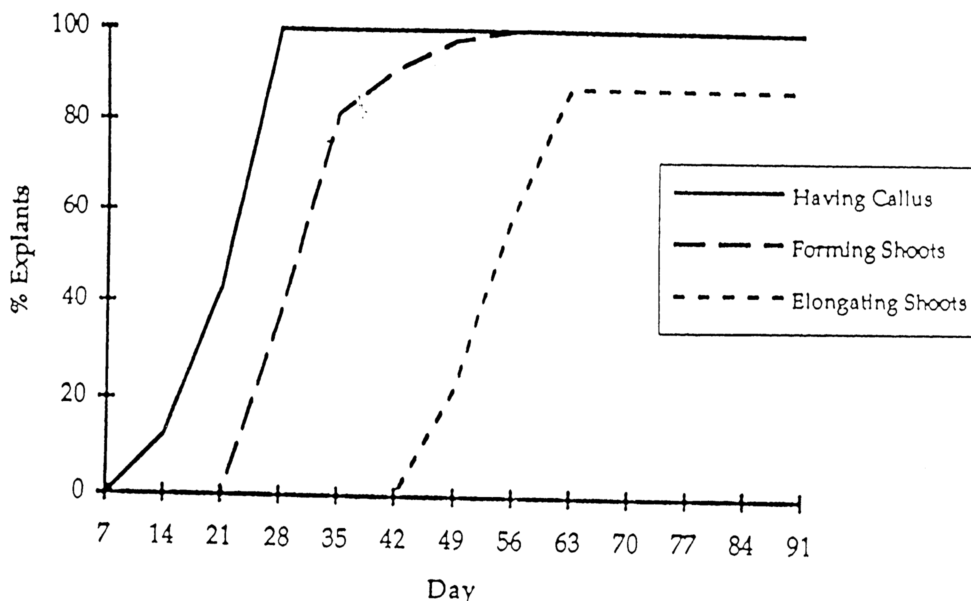
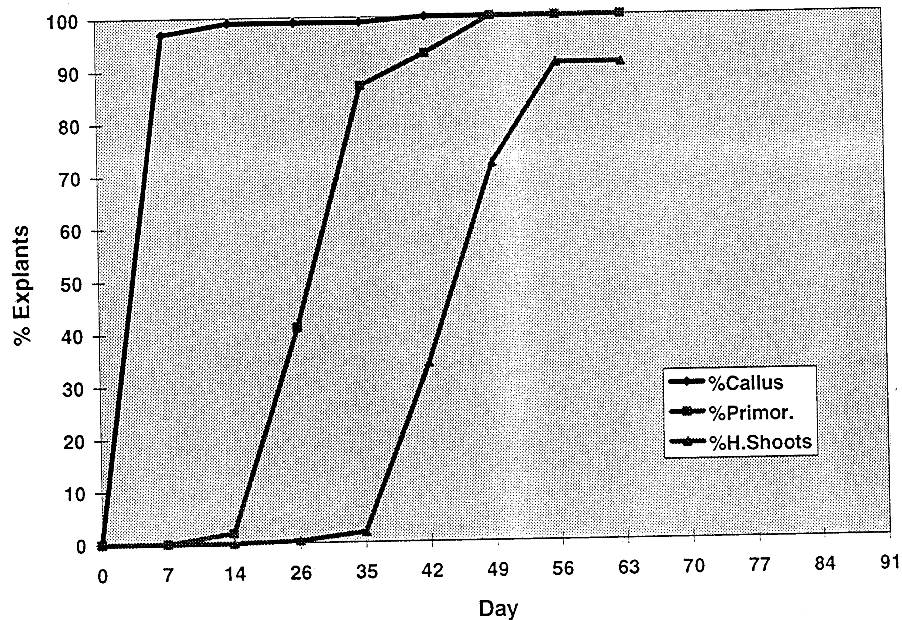


Figure 2.



In the second experiment, the treatment noted above was contrasted with a control (no growth regulators), direct exposure to SIM, and exposure to CIM for two other lengths of time (CIM-1 and -5). Individual treatments were applied to 10 internode explants in each of 5 or 10 petri plates (replications). Observations were recorded as noted above for the first trial, except that intervals between observations were different and that the experiment was terminated somewhat earlier. Callus, primordia, and harvestable shoot formation were summarized and expressed as percentages of explants per plate; harvestable shoot numbers were also summarized as mean yield per explant per plate. Data collected 126 days after start of culture were subjected to analyses of variance for the completely randomized design with five treatments and varying numbers of replications. Differences among means were evaluated via Duncan's Multiple Range Test. All tests of significance were done at P = 0.05.

Significant variation was found among treatments for all response variables except percent of explants forming primordia (Table 1). CIM treatments yielded significantly greater responses than other treatments in terms of harvestable shoot production, and longer CIM exposures gave the highest numbers of shoots per explant. Shoot formation tended to plateau at CIM-4; experience with greenhouse internodes suggests that performance could decline with longer exposures. Explants given the control treatment, as expected, were least productive. That this treatment produced nearly as many explants with primordia as the others, without the explants having formed callus, may result from elongation of preexisting structures. Dissecting internodes from nodes can be rather difficult, since plants derived from culture are small and have short internodes.

Table 1. Callus, primordia, and shoot formation on K417 internodes taken from culture. Days in culture = 126. Trial 2.

Media	Callus	Primordia	Shoots	Shoots per Explant
	------- % Explants with		-------	------ No. -------
Control	60a*	86	50a*	0.96a*
SIM	96 b	94	41a	1.00a
CIM-1	100 b	97	78 b	1.83a
CIM-4	100 b	98	90 b	6.20 b
CIM-5	100 b	96	94 b	6.36 b

* Means followed by different letters differ significantly from others in the same column at P = 0.05.

In sum, results from this and the exploratory trial clearly indicate that internodes from culture are suitable for use in transformation experiments, especially for clones not easily manipulated in the Leaf Section System.

Given the positive outcome with K417, trials have been launched with another model clone, C175. Preliminary results are similar to those for K417, but C175 may not be quite so responsive. Tests are also planned with three elite clones recently established in culture, and a transformation experiment using K417 internodes from culture is in progress.

SCREENING ANTIBIOTICS FOR IMPROVED TRANSFORMATION EFFICIENCY

Two experiments were completed to improve selection in and protection of transformed cultures.

The first trial, a lethal dose assay, examined response of greenhouse internodes from K417 to the selective antibiotic, kanamycin (K). The more known about sensitivity of various explants, the more explant types and therefore clones available for transformation trials. K concentrations were: 0, 25, 50, 100, 200, and 400 mg/L.

Callus, primordia, and shoot formation frequencies at K concentrations up to and including 100 mg/L were similar to or only slightly less than those of the control. At 200 mg/L, callus and primordia formation approached control frequencies, but shoot production was much reduced. At 400 mg/L, all development was prevented. Thus, K can be used to select transformed materials, when K resistance is conveyed by the construct in question, from greenhouse internode cultures, and such explants should be useful for transformation of clones not sufficiently responsive to other regeneration protocols. A higher K concentration (400 mg/L), however, must be used for selection than that used in the Leaf

Section System (50-150 mg K/L).

The second antibiotic experiment concerned protection of putatively transformed cultures from residual At contamination. Residual contamination by At, the bacterial agent used to effect transformation, is a serious issue. The more cultures lost to such contamination, the lower the probability of recovering transformed materials.

Earlier student research on transformation was plagued with residual At contamination (Bristol 1994). The antibiotic, carbenicillin (CA), routinely used to clear cultures is somewhat sensitive to light and temperature (David G. Thompson, Personal Communication), and therefore is likely to lose effectiveness over the long periods of time required for growth and development of cottonwood cultures. In addition, At is known to secrete an enzyme that partially degrades CA, another factor potentially lessening effectiveness.

Cefotaxime (CE) is effective against At, is considered more stable in culture, and is also more resistant to degradation by At (David G. Thompson). Most plant tissue cultures are not harmed by CE at concentrations up to 100 to 250 mg/L.

CE was tested as a potential substitute for CA in earlier student work (Shorter 1991), but results were unclear. Accordingly, new lethal dose assays were performed to determine if CE would kill At without disrupting development in cottonwood leaf section cultures.

CE concentrations tested in lethal dose assays included: 0, 25, 50, 100, 250, and 500 mg/L. To determine the dose sufficient to kill At, three petri plates containing LB media (Sambrook et al. 1989) supplemented with each of the above concentrations were prepared. Individual plates were inoculated with 100 uL of 1 X 10**9 cfu/mL, and cultured at 30 degrees C. Colony growth was evaluated at the end of the third and fifth days after start of culture.

Effects of these same concentrations on cottonwood cultures were examined by culturing C175 leaf sections in petri plates containing standard medium plus the antibiotic. Individual CE concentrations were represented by five plates, each containing five leaf sections. Frequencies of callus, primordia, and harvestable shoot formation were determined weekly through 91 days after start of culture.

At colonies formed and enlarged rapidly on control plates (0 mg CE/L). Concentrations of 100 mg/L or less inhibited At growth, but did not eliminate the bacteria. Growth, however, was stopped by concentrations of 250 and 500 mg/L.

After 91 days in culture, roughly half the length of a typical transformation experiment, callus and primordia formation was 100% regardless of CE concentration. Analyses of variance showed that

harvestable shoot production did not differ among CE concentrations (Table 2). Thus, CE will aid removal of residual At without inhibiting formation of callus, primordia, and harvestable shoots on C175 leaf sections.

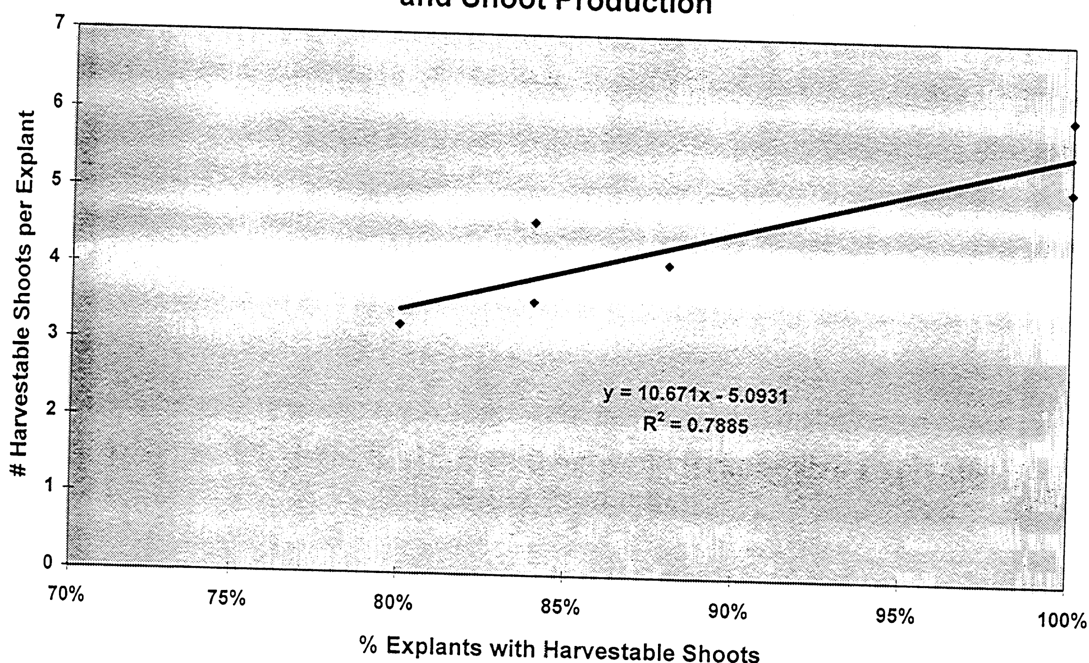
Table 2. Harvestable shoot production on C175 leaf sections as affected by cefotaxime concentration. Days in culture = 91.

Cefotaxime Concentration (mg/L)	Harvestable Shoot Production: Explants with Shoots (%)	Harvestable Shoot Production: Shoots per Explant (No.)
0	80*	3.24*
25	100	5.12
50	88	4.08
100	84	3.56
250	100	6.04
500	84	4.60

* No significant differences among means within columns at P = 0.05.

Results from this trial also confirmed a earlier perceived relationship between percent of explants with harvestable shoots and numbers of harvestable shoots per explant (Figure 3). In future, simple, quick counts of responsive explants can be used to decide if an experiment is likely to yield harvestable shoots in quantity. Savings in time and effort should be considerable.

Figure 3.



CE offers several advantages over carbenicillin, being less sensitive to light, less subject to inactivation by bacterial enzymes, and more stable in the lengthy culture times needed for cottonwood. Given these several findings and advantages, CE is being used in transformation trials (250 mg/L) in place of or in tandem with CA.

TRANSFORMATION, iaaM AND GUS REPORTER GENE EXPERIMENTS

Seven transformation experiments have been established since start of Fiscal Year 1994-95; two with the auxin synthesis (iaaM) gene and five with a benign marker gene (GUS Reporter Gene). Four trials, Trans 94-1 through 4, are discussed below in detail. Three trials with the GUS Reporter Gene, including one with K417 internodes from culture, were installed rather recently, and only preliminary results are available.

All experiments, unless noted otherwise, employed cottonwood model clone C175 and the IPST Leaf Section System. Trials with the iaaM gene utilized At Strain ASE (pTiT37SE) containing pMON604, a construct having a gene for K resistance linked to a strong constitutive promoter CaMV35S and the iaaM gene coupled to controllable heat shock promoter hsp70. The construct and At strain were provided by Monsanto Corp. Trials with the GUS Reporter Gene (GUS) used At Strain LBA4404 containing pBI121, a construct having a gene for K resistance coupled to the NOS promoter and GUS linked to CaMV35S.

Leaf section and transformation protocols were described in earlier publications/reports (Shorter 1991, Dinus 1992, Dinus and Stephens 1994). Any changes made to protocols for conduct of individual experiments are described in the context of individual experiments below.

TRANS 94-1, iaaM Gene.

This experiment was designed to repeat the transformation success achieved earlier, but with the iaaM gene coupled to a controllable promoter. Leaf sections were exposed to At (3 X 10**9 cfu/ml) for 5 min, rinsed and transferred to standard leaf section medium for three days of cocultivation. All leaf sections were then transferred to standard medium supplemented with 500 mg/L CA and/or 150 mg/L K. Treatments, numbers of petri plates (replications), and numbers of leaf sections were:

* No At and no K (-At-K). This treatment, represented by four petri plates each with five leaf sections, was used as a positive control. Objectives were to certify that leaf sections were responding normally, and to provide a base line against which to compare performance of other treatments.

* No At with K (-At+K). This kill control was included to confirm that 150 mg/L K was sufficient to stop development on and/or kill leaf sections. It used four plates, each containing

five leaf sections.

* Exposure to At without K (+At-K). This treatment was intended to permit leaf sections to develop normally (no K), with selection imposed later by transferring putatively transformed shoots to selective medium. Also, responses should mimic those of -At-K unless exposure to At or other factors associated with transformation interfere with development. Included were 41 petri plates with 5 leaf sections per plate.

* Exposure to At and K (+At+K). This treatment, immediate transfer to selective medium, provided for rigorous, rapid selection of transformants. Included were 41 petri plates with 5 leaf sections per plate.

Data were collected weekly through 77 days after start of culture, and at roughly monthly intervals thereafter. Development was quantified in terms of percentages of explants forming callus, primordia, and harvestable shoots. Putatively transformed materials were collected in later stages for DNA analyses (see below). Also, promising leaf sections were isolated from the +At+K treatment, and transferred to medium lacking K. This was done to foster development in the event that massive decline and/or death of tissues surrounding putatively transformed meristematic centers was inhibiting development.

Shoots were harvested when 0.5 cm or longer, regardless of treatment, pulsed on WPM + 0.1 mg/L IBA, and transferred for growth to Magenta boxes containing WPM +/- K. Half of all shoots harvested were transferred to selective medium (WPM+ 50 mg/L K), with the remaining half placed on nonselective medium (WPM-K). The latter served as controls, a check on whether or not transformation interfered with rooting and subsequent growth. Shoots surviving for four to six weeks were rerooted and transferred to WPM + 100 mg/L K. Observations on rooting, mortality, and external morphology were taken at approximately one month intervals. Leaves from shoots surviving on WPM+K and having morphological features symptomatic of excess auxin were collected for DNA analyses (see below).

Results through 77 days in culture indicate that control leaf sections (-At-K) developed callus, primordia, and shoots in accordance with past experience (Figure 4). Shoots formed and were harvested through 217 days in culture (Table 3). Leaf sections not exposed to At but cultured on medium containing kanamycin (-At+K) all died (Table 3), as expected for a kill control and confirming that 150 mg/L K is sufficient to eliminate nontransformed materials.

Figure 4.

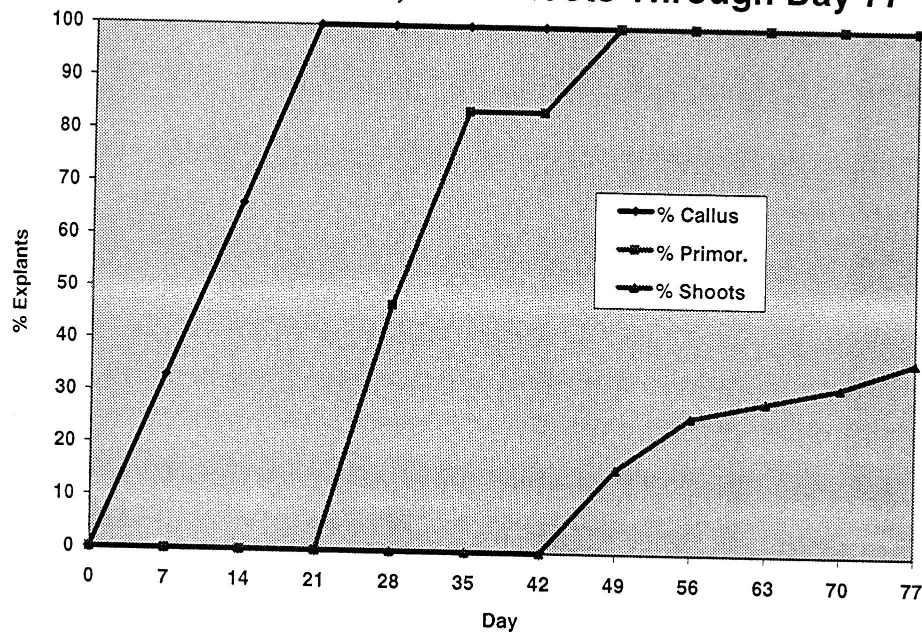
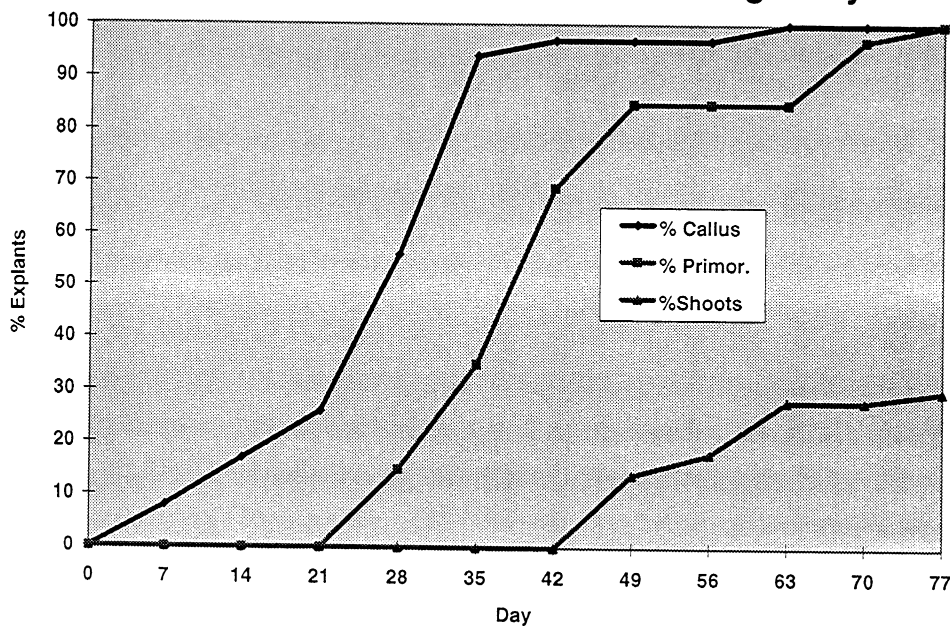


Figure 5.



Sections exposed to At but not K (+At-K) formed callus and primordia later than controls (Figure 5, but reached levels typical of control explants (Figure 4). Shoot formation, however, was reduced (Table 3). Even so, 335 shoots were harvested for rooting and evaluation on selective and nonselective media. As expected,

+At+K treatment caused rapid death of most leaf sections, with only small numbers forming callus and primordia (Table 3). No harvestable shoots were formed.

Residual At contamination was widespread; 89% of leaf sections exposed to At showed some signs of At growth at one time or another. Periodic rinsing and rescue operations, usually in conjunction with routine subculturing at 21-day intervals, prevented excessive loss of explants, but such levels clearly restrict efficiency and lower probability of achieving transformation. Replacing or supplementing CA with another more effective antibiotic is critical.

Table 3. Developmental status of C175 leaf sections from transformation trial 94-1 (iaaM gene) after 217 days in culture.

Treatment	Percent of Explants Forming		
	Callus	Primordia	Shoots
-At-K	100	100	100
-At+K	0	0	0
+At-K	100	100	47
+At+K	2	1	0

On day 77, 38 leaf sections from the +At+K treatment exhibited small areas of living tissue and some potential for further development. As noted above, these were transferred to nonselective medium to foster development of putatively transformed explants. By day 217, 32% retained some living tissue, 6% formed callus, and 2% developed primordia. Samples of callus and primordia were collected for DNA analyses (see below).

Shoots harvested from the -At-K (Control) treatment rooted and have grown well on WPM - K (Table 4). Shoots from this same treatment did not survive on WPM + K, suggesting that selection for transformants can be done at the shoot level. When the larger number of +At-K shoots are considered, however, the picture is not so clear (Table 4). Shoots on selective medium rooted at lower frequencies than those placed on nonselective medium, but the difference was only 20 percentage points (Table 4). Differential mortality also occurred, but effects of selective medium were not as great as desired. Large differences in rooting and mortality would indicate efficient selection, ie., WPM+K was quickly and effectively eliminating nontransformed shoots. This lack of differentiation is attributed to inadequate K (50 mg/L), or its degradation over time. A higher concentrations (100-150 mg/L) seems warranted.

Table 4. Status of shoots harvested from -At-K and +At-K treatments, rooted, and transferred to WPM +/- K for selection of transformants.

Original Treatment	Shoot Growth Medium WPM - K		WPM + K	
	% Rooting	%Mortality	%Rooting	%Mortality
-At-K (Control)	100	0	0	100
-At+K	72	20	52	33

Given these results, healthy shoots from populations described in Table 4 have been rerooted and transferred to WPM + 100 mg K/L. Early indications are that approximately 40% rooted again and continue to survive. Leaves have been collected for DNA analyses from the most vigorous individuals (see below).

As are most antibiotics, K is somewhat sensitive to light and temperature. Thus, culture environment and time may be lessening K effectiveness (Steve H. Strauss, Personal Communication). To ensure improved and more rapid selection, shoots harvested in future will be cultured on WPM + 150 K/L. Heightened selection efficiency may also be obtained by including K in the rooting pulse medium as well as the shoot growth medium.

Trans 94-3, iaaM Gene.

This experiment evaluated factors intended to heighten probability of transformation. The protocol used in the experiment described above was followed, with two exceptions. First, At cultures used in this research routinely are maintained and expanded in LB medium containing K, the selective antibiotic. This procedure ensures retention of the plasmid bearing the genes of interest. Presence of K during exposure of explants to At, however, could damage them, thereby lowering probability of generating and recovering transformants. In the current trial, At cultures were centrifuged for 5 min at 2500 rpm to settle cells, and permit decanting media containing potentially inhibiting antibiotics. Settled cells were washed with and resuspended in LB medium free of antibiotics. This suspension was then used for At exposure. Second, At exposure time and inoculum density were varied to determine if longer times and higher At doses would yield more transformants. Treatments included:

* No At or K (-At-K). This treatment was used to verify leaf section performance, and was represented by three petri plates (replications) each containing five leaf sections.

* Exposure to At, 1 X 10**7 cfu, for 5 min. Ten plates with five leaf sections each were given this inoculum density and exposure time. Subsequently, half of the leaf sections were transferred to

standard leaf section medium containing 150 mg/L K, and half to medium without K. This five min exposure was the same as used in the trial described above, thus allowing for comparisons among trials. As before, the -K option was included to permit normal leaf section development, with selection imposed later by transferring putatively transformed shoots to selective medium. Inclusion also allowed observation of whether or not the protocol interfered with development.

* Exposure to At, 1 X 10**8, for 5 min. Ten plates with five leaf sections each were used. We hypothesized that this increased At dose would yield more transformants than lower doses used in previous trials. Subsequent to exposure, half of the leaf sections were moved to +K and half to -K medium.

* Exposure to At, 1 X 10**7, for 30 min. Twenty plates with five leaf sections each represented this promising treatment; longer exposure times were hypothesized as being more likely to effect transformation than shorter ones regardless of At dose. Following exposure, leaf sections were divided equally between plates containing and not containing K.

* Exposure to At, 1 X 10**8, for 30 min. Ten plates with five leaf sections each were used to evaluate response to this higher At dose and long exposure. As for other treatments, exposed leaf sections were divided equally +K and -K subtreatments.

Observations were made weekly through 42 days after start of the trial to verify normal development, and at roughly 21 day intervals thereafter. Development continues on significant numbers of leaf sections, and observations are therefore continuing. Although additional data will accrue, observations through day 161 in terms of percentages of explants forming callus, primordia, and harvestable shoots have been summarized (Table 5) . Putatively transformed materials were collected, when and where available, for DNA assays.

Shoots were harvested as described for the preceding experiment, with observations on percent rooting, mortality, and external morphology taken every 21 to 30 days. Leaves from putative transformants were collected for DNA analyses.

Table 5. Developmental status of C175 leaf sections as affected by the varying At doses and exposure times used in transformation trial 94-3 (iaaM gene) after 161 days in culture.

Treatment			Percent of Explants Forming		
			Callus	Primordia	Shoots
-At-K:	Control		100	100	100
+At-K:	10**7,	5 min	100	100	100
		30 "	100	100	80
	10**8,	5 "	100	100	100
		30 "	88	88	72
+At+K:	10**7,	5 min	0	0	0
		30 "	0	0	0
	10**8,	5 "	0	0	0
		30 "	4	0	0

Results show that control (-At-K) leaf sections underwent normal development. Callus formation began 7-14 days after start of culture, and all explants eventually formed callus, primordia, and harvestable shoots (Table 5).

Centrifugation and washing to free At cells of antibiotics prior to exposing leaf sections had little effect. Development of leaf sections exposed to At and cultured on nonselective medium (+At-K) was slightly better than that observed in the aforementioned trial. That is, harvestable shoot production was greater, regardless of At dose or exposure time (Tables 3 and 5). Recovery of putatively transformed calli from explants cultured on selective medium (+At+K) was only slightly greater than when antibiotics were not removed by centrifugation and washing. Even though improvement was not great, the practice will be continued, as it is recommended and used by numerous authors. e.g., Clonetech Laboratories Inc., manufacturer of kits for genetic transformation.

Regardless of treatment, significant numbers of +At-K explants formed callus, primordia, and harvestable shoots (Table 5). Some variability occurred among treatments in that fewer leaf sections formed callus and primordia at the highest At dose and longest exposure time. Harvestable shoot production was also affected, with yields declining with increasing exposure time, regardless of At dose. This occurrence suggests that any advantage gained by greater doses and exposure times could be offset by interference with developmental processes. Countering any such interference necessitates using larger numbers of explants.

Development of leaf sections cultured on selective medium (+At+K) was halted for the most part (Table 5). No harvestable shoots were formed, and only four percent of these explants formed callus. Promising calli have been expanded to further development and to

provide sufficient tissue for DNA analysis. That the only calli produced in +K treatments were found in cultures receiving the longest At exposure and highest inoculum density may be coincidence. Such results, however, would seem expected, and future experiments will evaluate effects of these and even longer exposure times. Protocols used elsewhere rely on exposure times as long as four hours (Gerry A. Tuskin, Personal Communication).

Residual At contamination was considerable, although lower than in the preceding iaaM gene experiment. Contamination averaged over all treatments through 161 days was 75%, and was greater at the highest At dose and longest exposure times, especially for +K subtreatments. Rinsing and rescue operations done while subculturing every 21 days reduced losses, but whether or not this level of contamination, even if transient, interfered with recovery of transformants remains uncertain. Such findings provide additional impetus for identifying and using an antibiotic more effective than CA.

Shoots harvested from the several -K treatments rooted well (76%), with most making normal growth on nonselective shoot growth medium. Mortality to date has been only 27%. Performance on selective medium has been lower; rooting = 65% and mortality = 51%. Although greater than in the preceding experiment, differences between responses on selective and nonselective media still seem too small for effective selection to have occurred. Shoots surviving to date will be rerooted and transferred to shoot growth medium containing 150 mg/L. In the meantime, leaves for DNA analyses have been collected from the healthiest individuals surviving on selective medium (see below).

Trans 94-2, GUS Reporter Gene.

The purpose of this experiment was to transform C175 with a benign marker gene, the GUS Reporter Gene System manufactured by Clonetech Laboratories Inc. This construct and protocol provides a sensitive and versatile reporter enzyme for detecting transformation and gene expression. The GUS construct and Clonetech protocol were employed to effect transformation, and to evaluate the possibility that transformation attempts with the iaaM gene were limiting leaf section development and thereby preventing recovery of transformants.

The protocol recommended by Clonetech Laboratories Inc. was followed, with one exception. Several authors (e.g., Fillatti et al. 1987) recommend incubating leaf sections for 24-48 hr before exposure to At. The intent: Foster initiation of callus before dividing cells are challenged with At or selective medium. Accordingly, half of the leaf sections used in the current trial were incubated on standard leaf section medium for 24 hr prior to At exposure. Treatments included:

* No At and no K (-At-K). This treatment served to verify that explants were developing normally, and consisted of six petri

plates (replications), each containing five leaf sections.

* Exposure to At without K (+At-K). This treatment was installed, as in trials described above, to provide putatively transformed shoots for harvest, rooting, and later selection on shoot growth medium containing K. The treatment was also intended to supply information on whether or not the transformation process interfered with regeneration from leaf sections. Representation included 22 plates, each containing 5 explants.

* Exposure to At and K (+At+K). A total of 22 plates, each containing 5 leaf sections, was used to determine if immediate transfer to selective medium would yield transformants. As prescribed by Clonetech Laboratories Inc., selective medium contained only 50 mg/L K.

Data on percent of leaf sections forming callus, primordia, and harvestable shoots were collected weekly for the first 63 days of culture and at roughly 3-week intervals thereafter. Development is continuing, and data summaries (Table 6) reflect findings through 123 days. Putatively transformed calli and primordia have been collected for DNA analyses.

In addition, 65% of the +At+K leaf sections had small areas of living tissue, and appeared to have the potential for further development. Half of these explants were isolated on the 49th day of culture and transferred to standard medium lacking K. This was done to stimulate further development in the event necrotic tissue surrounding the small clusters of living cells was interfering with callus, primordia, and shoot formation.

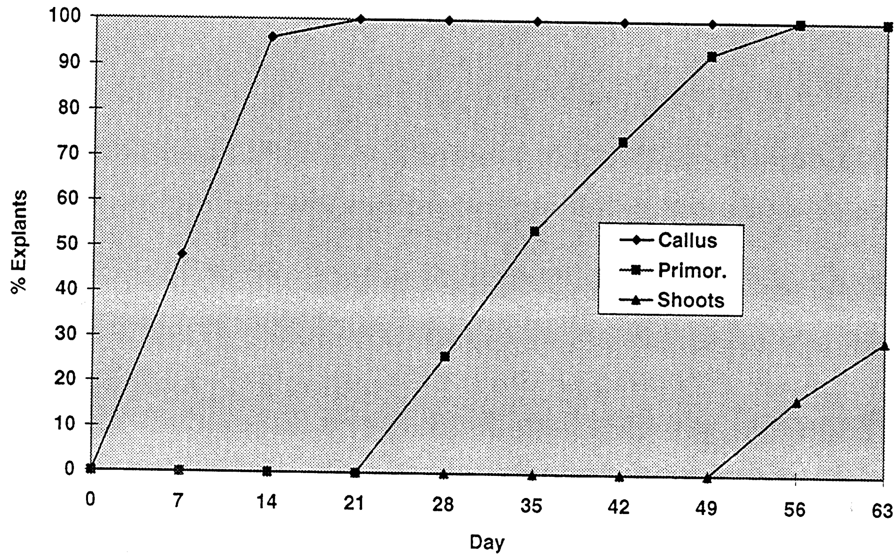
As in the preceding trials, shoots were harvested from +At-K leaf sections, rooted, and transferred to selective (50 mg K/L) and nonselective shoot growth medium. Transfers were completed only recently and results are not yet available. Even so, leaves have been collected from particularly promising individuals for DNA analyses (see below).

Results from this trial showed that incubation on standard leaf section medium for 24 hr prior to At exposure provoked no differential responses. Accordingly, results given below are averaged all explants given a particular treatment.

In retrospect, this finding is not surprising. To be effective, any such treatment should be sufficiently long to ensure that development is not only started but well underway. Examination of the course of development on leaf sections over time (e.g., Figures 4 and 6) shows that callus formation normally begins in the first few days of culture. Appearance of primordia, first visible manifestation of organized meristematic centers, however, occurs 14 to 21 days later. Thus, incubation to ensure that development is not hindered by exposure to At and/or selective medium probably should span 14 to 21 days after the start of culture. In addition, such treatments most likely are best applied after At exposure but

before transfer to selective medium. This would provide freshly wounded surfaces for At attack, allow more time for transformation to occur, and permit transformed cells to multiply before dying cells surrounding them can interfere. Thus, incubation for 14 to 21 days after At exposure will be tested in future.

Figure 6.



Summarized over incubation treatments, weekly observations through 63 days in culture show that control explants (-At-K) performed roughly as did those in previous trials. Callus formation began early (Figure 6), but reached 100% somewhat later than in earlier trials (Figure 4). Primordia likewise formed later and increased slower than expected (Figures 4 and 6). All explants nevertheless formed primordia by day 56. Shoot formation lagged even more so, but continued to increase until approaching 100% by day 123 (Table 6). All told, explants given this treatment performed well, but not as well as usual. Thus, any departure from expectation in other treatments may be more a function of explant condition and responsiveness than of treatment effects.

Development of +At-K leaf sections was delayed considerably relative to control treatments in this and preceding experiments (Figure 4, 6, and 7). This seems a normal outcome, but some delay also occurred relative to comparable treatments (Figures 5 and 7). Percentages of explants forming callus and primordia eventually reached expected levels (Table 6), but shoot formation was both delayed and reduced relative to earlier trials.

Figure 7.

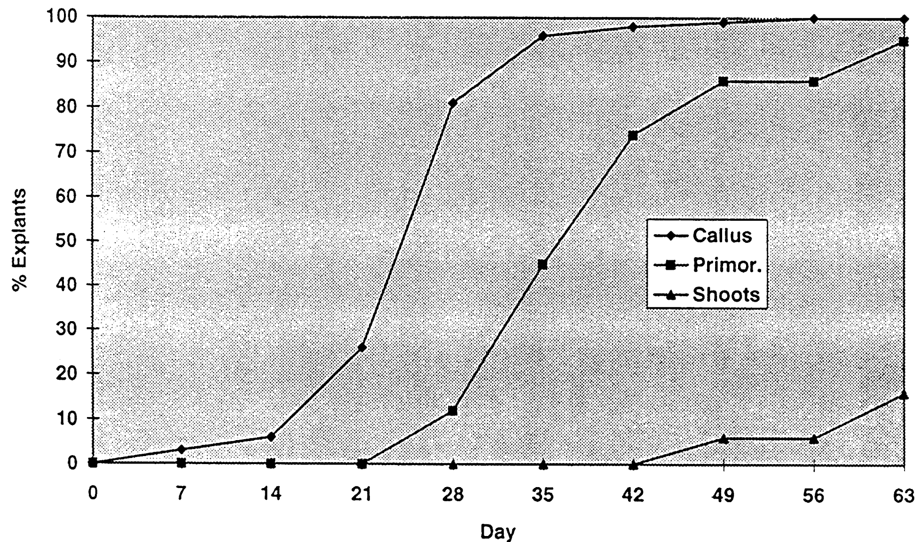
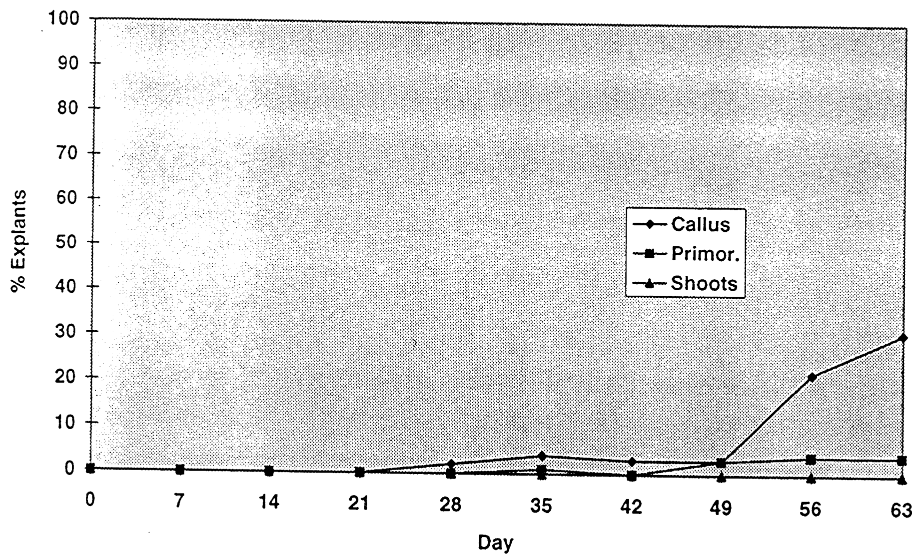


Figure 8.



As expected, development of explants given the +At+K treatment was slow. Significant numbers of explants did develop callus, primordia, and shoots (Figure 8, Table 6). Although encouraging at first glance, such results may be cause for concern. Selective medium in the GUS protocol includes only 50 mg K/L, and some putative transformants identified on selective medium could be escapes. Shorter (1991) demonstrated that 30 mg/L K was sufficient to stop development of or kill C175 leaf sections. The dose used in the current trial, 50 mg/L, should therefore be adequate to

eliminate nontransformed material. Nevertheless, some developing tissues could be shielded from K by the mass of dying tissues around them. In addition, K effectiveness could decline over the 21 days between subcultures. Thus, future trials should be executed with higher K levels or perhaps with stepped levels, starting at 50 mg/L and increasing to 150 mg/L over several subculture periods.

Table 6. Developmental status of C175 leaf sections 123 days after exposure to At containing the GUS Reporter Gene in transformation trial 94-2.

Treatment	Percent of Explants Forming		
	Callus	Primordia	Shoots
-At-K	100	94	94
+At-K	100	97	19
+At+K	31	5	1

On the 49th day of culture, 30 leaf sections given the +At+K treatment exhibited some promise or degree of development. As noted above, 30 such sections were transferred to nonselective medium to stimulate further development. On day 49, only 7% of these 30 explants had callus and/or primordia. By day 123 or after 74 days on nonselective medium, 67% had callus and 10% had primordia. Although transfer to nonselective medium may have fostered growth of escapes, tissues from the seven most vigorous explants have been isolated for GUS and DNA analyses (see below).

Residual At contamination was far less of a problem in this experiment. Cumulative contamination averaged only 11%, a level dramatically lower than in numerous trials conducted during the last several years. Although causes are uncertain, this outcome most likely results from lower aggressiveness of the At strain used in the GUS protocol.

Results concerning rooting and mortality of harvested shoots will be reported in future. Leaves from especially vigorous individuals growing on shoot growth medium supplemented with K have been collected for GUS and DNA analyses (see below).

Trans 94-4, GUS Reporter Gene.

This experiment was designed to test effects of CA and CE on residual At contamination, and to determine if substituting CE for CA affects GUS transformation of C175.

The GUS protocol recommended by Clonetech Laboratories Inc. was followed, with one alteration. Half of the leaf sections were cultured on standard leaf section medium supplemented with 500 mg/L

CA and half on the same medium with 250 mg/L CE. Treatments included:

* No At and no K (-At-K). This treatment served to track and verify explant development, and consisted of six petri plates (replications), each containing five leaf sections.

* Exposure to At without K (+At-K). This treatment served, as in trials described above, to provide putatively transformed shoots for later selection on shoot growth medium containing K. Results should also verify earlier findings that CE does not interfere with regeneration from C175 leaf sections. Representation included 30 plates, each containing 5 explants.

* Exposure to At and K (+At+K). A total of 30 plates, each containing 5 leaf sections, was used. The purpose was to determine if immediate transfer to selective medium would yield transformants, and to document any differential effects of CE and CA on yield.

Percent of leaf sections forming callus, primordia, and harvestable shoots were recorded weekly for the first several weeks of the trial. Special attention was given to establishing that development of explants from the various treatments was proceeding normally. Observations continued thereafter at roughly 6-week intervals. The experiment is still in progress, and data summaries (Table 7) reflect findings through only 115 days. Putatively transformed materials have been collected when and where available for GUS and DNA assays.

Similarly, shoots have been harvested from +At-K explants for rooting and transfer to selective (50 mg/L) and nonselective shoot growth medium. Although results cannot yet be assessed with accuracy, leaves have been collected from the healthiest individuals for GUS and DNA analyses (see below).

Explants from the control treatment (-At-K) began forming callus, primordia, and harvestable shoots early in the experiment. Frequencies reached or approached 100% (Table 7), and were similar for CA and CE treatments, confirmation that CE does not interfere with regeneration from C175 leaf sections.

Performance of leaf sections exposed to At without K (+At-K) was quite similar to that of controls (Table 7), except that shoot production was somewhat lower. Extent of the reduction, however, was less than or in line with declines observed in earlier trials. Differences between responses of explants grown on CA and CE were nominal.

Explants exposed to At and K (+At+K) also formed callus and primordia with considerable frequency (Table 7). Indeed, results were on a par with or better than those from the GUS trial described above. Shoot formation was similar or slightly higher than that noted in the earlier trial. No differences were apparent

between responses to CA and CE. The overall high levels of response to this treatment may indicate that K concentration in the selective medium is too low for effective selection, and that some surviving tissues, differentiated and nondifferentiated, are escapes. The results nevertheless are encouraging, and frequency of transformation will be confirmed via GUS and DNA assays. In future, adjustments will made to diminish probability of escape by using higher K levels or perhaps increasing K levels over time, ie., starting with 50 mg/L and increasing to 150 mg/L over several subculture periods.

Table 7. Developmental status of C175 leaf sections 115 days after exposure to At containing the GUS Reporter Gene and cultured on medium containing 500 mg/L carbenicillin and 250 mg/L cefotaxime in transformation trial 94-4.

Treatment	**Percent of Explants Forming**		
	Callus	**Primordia**	**Shoots**
Carbenicillin			
-At-K	100	100	100
+At-K	97	94	70
+At+K	33	11	3
Cefotaxime			
-At-K	100	100	93
+At-K	100	99	65
+At+K	33	7	0

Residual At contamination was not as severe a problem in this trial as in experiments with the iaaM gene, but was several fold greater than in the earlier GUS trial. Midway through the experiment, percentages of infested plates ranged from 43 to 60%, with more plates from the +At+K treatments being contaminated than others. Differences between CA and CE treatments were minor. By the 115th day of culture the margin between antibiotics had widened, particularly for the +At+K treatment. Within this treatment, 57% of leaf sections cultured on CA experienced At contamination at one time or another during the trial, whereas only 39% of those given CE were affected. Lower contamination relative to iaaM gene trials most likely results from lesser aggressiveness of the At strain used in the GUS protocol, but reasons for fluctuations among GUS trials are not known. These uncertainties aside, CE provided better control of residual At contamination without detrimentally affecting C175 leaf sections. Findings from the current trial therefore reinforce the decision to use CE in future transformation experiments.

Data on rooting and mortality of harvested shoots will be reported in the future. Leaves from promising plants transferred to WPM + K will be collected for GUS and DNA assays as the plants reach appropriate size.

CONFIRMATION OF TRANSFORMATION

Survival of callus, primordia, and/or shoots on selective medium infers that transformation has been effected. Confirmation, however, must be obtained by other independent means, e.g, by documenting GUS gene expression.

For confirmation then, samples of putatively transformed plant materials were withdrawn from each of the four experiments described above for screening by a DNA polymerase chain reaction (PCR) technique for the iaaM gene and a histochemical method for GUS expression (Jefferson 1987). Plant material included:

* Calli and/or primordia from leaf sections cultured on selective medium containing either 150 mg/L K (iaaM gene trials) or 50 mg/L K (GUS gene trials); and

* Leaves from the more vigorous plants rooted and surviving on selective medium (WPM + 50 mg/L K).

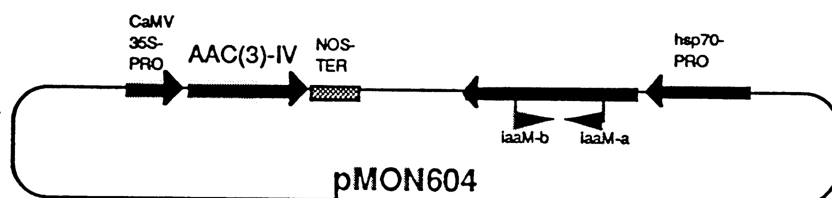
Tobacco plants transformed earlier with the same GUS construct used in the C175 experiments served as a control for GUS assays. Positive reactions with tobacco were taken as an indication that reagents and procedures were functional in tests of putative C175 transformants.

Constructs and primers for PCR analyses were obtained as outlined below. The plasmid pMON604 as originally constructed at Monsanto Corp. contains the iaaM gene flanked by maize heat shock promoter hsp70 and the ACC(3)-IV gene for K resistance driven by the CaMV35S promoter (Figure 9). The At strain used at IPST for transformation is a derivative of C58 which contains a disarmed nopa-type Ti plasmid, pTiT37-SE. Sequence specific primers were designed and made to amplify a region of 1.17 kb of the iaaM gene (Figure 9). Primer sequences are:

iaaM-a 5′ GTGAGTGGTCTCGTTGCCATCGAC 3′; and

iaaM-b 5′ TGGCATATGCGCTGGCTCACAGAC 3′.

Figure 9. Schematic diagram of the pMON604 plasmid showing location of relevant genes and promoters.



For DNA isolation, individual calli or leaves were placed in 1.7 mL microcentrifuge tubes. Loaded tubes were flash frozen with liquid nitrogen, and the contents ground to a powder with a stirring rod. Two µL of hot (60-65°C) extraction buffer (Chang et al. 1991) was added to the tubes, which were then incubated in a water bath of the same temperature for 15 min. The mixtures were then extracted twice with chloroform:isoamylalcohol (24:1), and DNA precipitated from the supernatant by adding 2 volumes of ethanol. After incubation of these mixtures at -20°C for at least 2 hr, DNA was pelleted by centrifuging at 13,000 rpm for 10 min. DNA pellets were washed with 75% ethanol, dried under vacuum, and were then dissolved in water. DNA quality and quantity were checked by electrophoresis.

Approximately 100 ng of DNA was used as a template for PCR amplification of iaaM gene fragments. PCR reaction mixtures contained 1X buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1% Triton X-100, Mg-free), 250 nM of dNTPs, 1.5 mM MgCl2, 100 pmoles of each primer, and 2 units of Taq DNA polymerase. PCR operating conditions were set as follows: Denaturation for 5 min at 94°C; 35 cycles at 3 temperature settings (1 min at 94°C followed by 1 min at 60°C and 1 min 20 sec at 72°C); and finally 5 min at 72°C.

The PCR or DNA amplification technique outlined above produces manifold copies of unique regions as delineated by the primers. In this particular case, the amplified region is a 1.17 kb region of the iaaM gene known to be specific to the At-derived construct used for transformation. Detection of the amplified region, if present in DNA from putative transformants, is accomplished by transferring products from the PCR technique to agarose gels, separating them via electrophoresis, staining with ethidium bromide, and visualization under uv light.

For histochemical assays of GUS gene expression, individual calli or leaves were transferred to wells of a 96-well assay plate. Calli were processed without the usual fixation step. Leaves were fixed with 2.5% glutaraldehyde in 0.1 M NaPO4 (pH 7.0). Plates were held on ice for 2-3 min, after which the fixative was removed and leaves rinsed briefly with 0.1 M NaPO4 buffer. To complete the assays, calli or fixed leaves were incubated overnight at 37 degrees C in 1.5 mM of X-GLUC plus 0.1 M NaPO4 buffer. Following incubation, tissues were washed with absolute ethanol until all green coloration was removed, and then observed via microscope for presence or absence of the blue coloration produced by GUS expression. The GUS or uidA gene codes for β-glucuronidase, an enzyme which converts colorless 5-bromo-4-chloro-3-indolyl glucuronide (X-GLUC) into a stable blue indigo colored dye (Jefferson 1987).

Results of analyses described above confirmed successful transformation with both the iaaM and GUS genes. Five calli taken from the first iaaM trial proved positive for presence of the iaaM gene (Table 8, Figure 9). Two calli from each of two experiments gave positive reactions for GUS gene expression (Table 9).

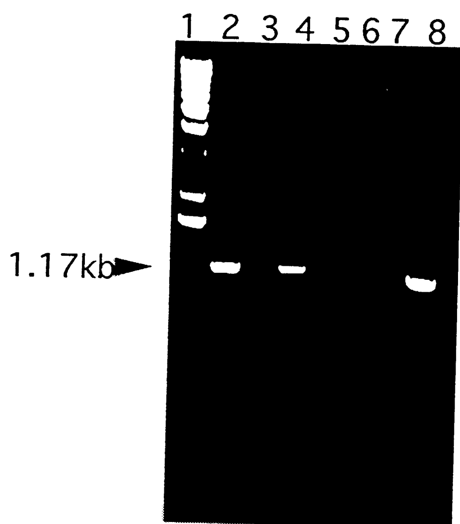
Table 8. Numbers of putative transformants tested for and confirmed as showing presence the iaaM gene via the PCR technique.

Experiment	Calli, No.		Shoots, No.	
	Putative	Confirmed	Putative	Confirmed
Trans 94-1	7	5	19	0
Trans 94-3	ND*	ND*	18	0

* No Data Collected.

Calli confirmed as containing the iaaM gene were all derived from the +At+K treatment, and had been transferred to nonselective medium on Day 77 to foster development of either calli or small, promising areas of living tissue. That this action produced transformants demonstrates utility of allowing development to proceed, free of selection pressure, for some portion of the developmental cycle. Allowing development to start before selection may be even more effective, and new trials have been established to evaluate the impact of culture on nonselective medium for roughly the time required for callus formation. Future trials will also test short periods of time on selective medium, followed transfer to nonselective medium and back to selective medium. On a per leaf section basis, the five calli confirmed as transformed with the iaaM gene represent a 2.4% yield.

Figure 10. PCR confirmation of C175 transformation with the iaaM gene. Lanes are: KB DNA size ladder (1); products from using pMON604 plasmid DNA as template (2); control plant DNA as template (3); DNA from five calli collected from Experiment TRANS 94-1 as template (4-8).



Putatively transformed calli from the second iaaM gene trial formed at higher frequency than in the first trial; increasing At concentrations and exposure times had been hypothesized as likely to raise transformation efficiency. None of these calli, however, were transferred to nonselective medium for improved development as was done in the first experiment, and growth to date has been too slow to permit PCR assay. Efforts to stimulate growth and development have been implemented, and PCR analyses will be performed as feasible.

Table 9. Numbers of putative transformants tested for and confirmed as showing GUS gene expression via histochemical assay.

Experiment	Calli, No.		Shoots, No.	
	Putative	Confirmed	Putative	Confirmed
Trans 94-2	16	2	60	0
Trans 94-4	19	2	3	0

As in the iaaM experiments, calli testing positive for GUS gene expression all came from +At+K treatments. Not all putatively transformed calli, however, have been assayed; only the largest and most vigorous samples have been tested to date. Analyses are ongoing, and percentages of confirmed transformants per treatment and/or trial have not been computed. Final determinations will be made only after all promising cultures have been evaluated. Calli confirmed as transformants are being cultured to stimulate growth; subsamples will be checked to verify permanence of transformation and others will be transferred to media, nonselective and selective, to stimulate formation of primordia and/or shoot elongation. Several measures for overcoming inhibition of development by K are also being considered; e.g., a somewhat lower K concentration (100 versus 150 mg/L) and coupling of a stronger promoter to the gene for K resistance.

That at least a few shoots harvested from +At-K treatments in the four experiments were not transformed is most disappointing. Modest numbers of shoots harvested from all trials not only rooted but also have grown well for two or more months on shoot growth medium plus 50 mg/L K. C175 leaf sections, treated properly, can produce large numbers of shoots, and identifying the 1-3% that are transformed via selection on shoot growth medium supplemented with K or by PCR analyses is laborious. Efforts to improve selection efficiency by raising K concentrations to 150 mg/L at time of rooting or by stepped increases from 50 to 150 mg/L are underway. Any surviving this continued selection pressure will be checked for transformation via PCR and/or GUS assay.

In GUS trials, several leaf sections formed shoots even on selective medium. In addition, numerous shoots from +At-K treatments rooted, survived, and grew rapidly after transfer to

shoot growth medium containing K. That none of these expressed GUS gene activity may indicate differential expression, ie., the CaMV35S promoter may not function in stem or leaf tissues of C175. To account for this possibility, a PCR technique is being developed to evaluate these and other shoots for transformation.

ACKNOWLEDGEMENTS:

In addition to IPST member company funds, this research was supported by a grant of $27,940 from The Georgia Consortium for Technological Competitiveness in the Pulp and Paper Industry.

Also acknowledged is the kind technical assistance provided by Mr. Vincent T. Ciavatta (M.S. Student, IPST), Ms. Eida Y. Green (Research Intern, Westlake High School, Atlanta), and other members of the IPST Forest Biology Technical Staff.

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Research Proposals Submitted

RESEARCH PROPOSALS
(Submitted and in Review or Submitted and Granted)

Following is a list of 1994/1995 research proposals which have been submitted and awarded or are currently under review. Following the list is a brief abstract or summary from each proposal. If you would like to review any of these proposals please contact one of the IPST authors.

Title:	Protecting Georgia Forests through Biotechnology
Authors (Affiliation):	John Cairney (IPST), Sarah Covert (UGA), Scott Merkle (UGA), Gerald Pullman (IPST)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$ 252 976 ($ 163 476 to IPST)
Period of Proposal:	1996-97
Submitted:	1st July 1994
Status:	In Review

Title:	Gene Expression in Staged Zygotic and Somatic Embryos of Loblolly Pine
Authors (Affiliation):	Gerald Pullman (IPST), John Cairney (IPST), Jung H. Choi (GIT)
Awarding Agency:	Georgia Institute of Technology/Institute of Paper Science and Technology, Pulp and Paper Education Program
Amount Requested:	$ 40 000 ($ 20 000 to IPST)
Period of Proposal:	1994-1995
Submitted:	15th July 1994
Status:	**Grant Awarded**

Title:	Improved Fibers for Pulp and Paper Production Through Genetic Engineering of Southern Tree Species
Authors (Affiliation):	Jeffrey F.D.Dean (UGA), Karl-Erik L. Eriksson (UGA), Scott A. Merkle (UGA), Ronald J. Dinus (IPST) and John Cairney (IPST)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$ 130 969 ($ 58 789 to IPST)
Period of Proposal:	1994-96
Submitted:	1st July 1994
Status:	Request for continuation of research for 1993-1994 and 1994-1995 have already been granted. The request for 1995-1996 funds is in review.

Title:	Pollution and Water Deficit-Inducible Gene Expression: Glycine-Rich Proteins
Authors (Affiliation):	Ronald J. Newton (Texas A&M), Alesia J. Reinisch (Texas A&M), John Cairney (IPST), Richard B. Flagler (Texas A&M)
Awarding Agency:	EPA
Amount Requested:	~$ 200 000 ($ 10 000 Consultancy to IPST)
Period of Proposal:	1994-1996
Submitted:	15th July 1994
Status:	In Review

Title:	Pollution Preventation in Paper Mills: Reducing Pulping Waste Through Genetic Engineering of Lignin Synthesis
Authors (Affiliation):	John Cairney (IPST), Gerald Pullman (IPST), Ronald Dinus (IPST), Shujun Chang (IPST), Art Ragauskas (IPST), Ronald Newton (Texas A&M University), John Ralph (USDA-ARS, Madison, WI)
Awarding Agency:	EPA (Environmental Technology Initiative)
Amount Requested:	$562 478 ($ 379 500 to IPST)
Period of Proposal:	1995-97
Submitted:	21st September 1994
Status:	In Review

Title:	Enzymatic Deinking of Recycled Office Waste.
Authors (Affiliation):	Chuck Corchene (IPST), Ron Dinus (IPST), and Karl-Erik L. Eriksson (UGA)
Awarding Agency:	EPA
Amount Requested:	$221,380
Period of Proposal:	1995-97
Submitted:	21 st September, 1994
Status:	In Review

Title:	Fast-Growing Trees for Carbon Recycling, Energy and Wood Production, and Rural Development.
Authors (Affiliation):	Ron Dinus (IPST), Gerald Pullman (IPST), John Cairney (IPST), Scott Schlarbaum (Univ. Tennessee), Russell Cox (Tennessee Division of Agriculture - Division of Forestry), Scott Merkle (UGA).
Awarding Agency:	EPRI
Amount Requested:	$250,000
Period of Proposal:	1995-1997
Submitted:	Preproposal submitted December 14, 1994
Status:	In Review

Title:	Acquisition of Biotechnology Equipment for Forest Tree Improvement
Authors (Affiliation):	Gerald Pullman (IPST), John Cairney (IPST), Ron Dinus (IPST)
Awarding Agency:	NSF
Amount Requested:	$224,698
Period of Proposal:	1995-97
Submitted:	March 1st, 1995
Status:	In Review

The Georgia Consortium for Technological Competitiveness in Pulp and Paper

1. Title: Protecting Georgia Forests through Biotechnology

2. Institutions: Institute of Paper Science and Technology (IPST)
University of Georgia, Athens (UGA)

3. Investigators:

Co-Principal Investigator: **Dr. John Cairney**
Department of Forest Biology
Institute of Paper Science and Technology
500 10th Street NW
Atlanta GA 30318
Tel: (404) 894 1079
Fax: (404) 853 9510

Co-Principal Investigator: **Dr. Sarah Covert**
School of Forest Resources
University of Georgia
Athens GA 30602
Tel: (706) 542 1205
Fax: (706) 542 8356

Associate Investigator: **Dr. Scott Merkle**
School of Forest Resources
University of Georgia
Athens GA 30602
Tel: (706) 542 6112
Fax: (706) 542 8356

Associate Investigator: **Dr. Gerald Pullman**
Department of Forest Biology
Institute of Paper Science and Technology
500 10th Street NW
Atlanta GA 30318
Tel: (404) 853 1871
Fax: (404) 853 9510

4. Executive Summary

Few people deny that biotechnology will play an important role in the forest industry of the future. Nevertheless, relatively few scientists world-wide are developing research programs that apply molecular biology to forestry related problems. To advance the economic interests of Georgia forestry, a foward-thinking research program that focuses on problems relevant to the forest industry in Georgia must be initiated. Future benefits lie not only in the creation of trees that are specifically designed to thrive in Georgia's climate, but also in the licensing of methodologies that are likely to be applicable to a wide-range of problems. Furthermore, much as biotechnology jobs have been created near centers of learning in California and Massachusetts, promotion of forest biotechnology within Georgia's research institutions may lead to the development of a local forest biotech industrial community. With established research programs at the University of Georgia (UGA) and the Institute of Paper Science and Technology (IPST), a good opportunity exists for Georgia to step to the national fore-front of forest biotechnology research.

The continued and future success of Georgia forestry will depend in part on shielding forest trees from biological and environmental stresses which are responsible for millions of dollars of losses annually in commercial Georgia forests. Our ability to ameliorate the impact of pathogenic and climatic disturbances is limited by a lack of knowledge on these topics. A potential solution, however, may come through the modern techniques of molecular biology. Such techniques offer great insights into the physiology of stress response in trees. Research shows that plants respond to environmental stress by activating genes whose products function in repair and defense. In agronomic crop species some of these genes have been cloned, modified and re-introduced into plant cells to produce transgenic plants with enhanced tolerance to viruses, insect attack, herbicides, and salt stress. We intend to apply this crop impovement approach to forest species.

We propose to study drought and fusiform rust infection as two important stresses that affect Georgia forests. The joint consideration of drought and pathogen attack is merited because each stress renders plants susceptible to the damaging effects of the other. We hypothesize that trees resistant to these stresses can be created through the following genetic engineering approaches. Pine genes that are activated by drought conditions have already been identified (Cairney et al. 1993). To determine if the proteins made by these genes help protect trees against drought, they will be expressed at high levels in a species that can be manipulated with relative ease, yellow-poplar. In addition, the promoter elements necessary for the drought activation of one of these genes will be identified.

Pine genes activated by fusiform rust infection will be harnessed to create rust resistant pines. Molecular genetic techniques will be used to identify pine genes that are induced by fusiform rust infection. The promoters controlling expression of these genes will be attached to a gene encoding a cytotoxin (i.e. barnase, an extracellular RNA-degrading enzyme) and transformed into rust-susceptible pine trees. It is hypothesized that when such transgenic trees are infected by *Cronartium*, the cytotoxin will be produced at the site of infection, neighboring pine cells will be killed and the establishment of a fusiform rust infection will be blocked. Due to time constraints the creation of transgenic pines is beyond the scope of this proposal, however, the initial steps of this protection scheme, up to pine transformation, will be completed.

An essential part of a scheme of producing genetically engineered pines is the ability to produce plantlets from cells in culture. The low efficiency of the process currently hinders forest biotechnology. Insights into and improvements in somatic embryogenesis will be pursued by expansion of a successful program at IPST and analysis of media composition and additives will be further evaluated.

These two projects represent novel aproaches to the genetic improvement of southern softwood and hardwood species and hold enormous potential for improving forest health and productivity. In addition, this research will further develop expertise already available at two Georgia institutions, IPST and UGA.

Key Words: Drought, Fusiform Rust, Biotechnology, Tolerance, Stress-related Genes, Transgenic Plants

FY 1994-95 Request For Seed Grand Application
Georgia Institute of Technology/Institute of Paper Science and Technology
Pulp and Paper Research and Education Program

Gene Expression in Staged Zygotic and Somatic Embryos of Loblolly Pine

Co-Principal Investigators: Dr. Gerald Pullman
Department of Forest Biology
Institute of Paper Science and Technology
500 10th Street NW
Atlanta, GA 30318
Tel: (404) 853-1871
Fax: (404) 853-9510

Dr. John Cairney
Department of Forest Biology
Institute of Paper Science and Technology
500 10th Street NW
Atlanta, GA 30318
Tel: (404) 894-1079
Fax: (404) 853-9510

Dr. Jung H. Choi
School of Biology
Georgia Institute of Technology
Atlanta, GA 30332-0230
Tel: (404) 894-8423
Fax: (404) 853-0048

Funding amount Requested: $40,000

Executive Summary: A continued supply of low-cost, high quality raw materials is essential for the future success of the U.S. Forest Products Industry. The clonal propagation of high value trees through somatic embryogenesis has the potential to help meet future industry needs by increasing forest yields and improving raw material uniformity and quality. Somatic embryos of loblolly pine can be produced currently but quality is inferior when compared to natural seed embryos. In this study we propose to conduct the first comprehensive biochemical analysis of embryo development attempted in conifers and one of very few attempted in plants. Zygotic and somatic embryo development will be classified into specific stages from which proteins, m-RNAs, and amino acid levels peculiar to these stages will be identified. The identified stage specific nutritional requirements and markers will be applied to the improvement of embryo maturation and the further study of embryo development.

Co-Principal Investigators' Signatures:

Gerald Pullman

John Cairney

Jung H. Choi

Administrative Officers' Signatures:

R. G. Makowski

Title: President-IPST

Harry Poehlein

Title: VPIP/GIT

THE GEORGIA CONSORTIUM FOR TECHNOLOGICAL COMPETITIVENESS IN PULP AND PAPER

FIBER SUPPLY DIVISION
FY 96 PROJECT APPLICATION
FINAL PROPOSAL

1. **TITLE:** **Improved Fibers for Pulp and Paper Production Through Genetic Engineering of Southern Tree Species**

2. **INSTITUTIONS:** University of Georgia, Athens (UGA)
Institute of Paper Science and Technology (IPST)

3. **INVESTIGATORS:**

Jeffrey F.D. Dean	Department of Biochemistry, University of Georgia, Athens, GA 30602-7229 (706) 542-7640 (706) 542-2222 FAX
Karl-Erik L. Eriksson	Department of Biochemistry, University of Georgia, Athens, GA 30602-7229 (706) 542-7640 (706) 542-2222 FAX
Scott A. Merkle	Warnell School of Forest Resources, University of Georgia, Athens, GA 30602 (706) 542-6112 (706) 542-8356 FAX
Ronald J. Dinus	Forest Biology Group, Institute of Paper Science and Technology, 500 10th St., Atlanta, GA 30318 (404) 853-9500 (404) 853-9510 FAX
John Cairney	Forest Biology Group, Institute of Paper Science and Technology, 500 10th St., Atlanta, GA 30318 (404) 853-9500 (404) 853-9510 FAX

4. EXECUTIVE SUMMARY

New techniques in genetic engineering and plant tissue culturing have provided the means for rapid acceleration of forest tree improvement. We have assembled a collaborative research team to focus the latest techniques of modern biotechnology and genetic engineering on the potentials and problems inherent to Georgia's commercial forest tree species in an effort to preserve and improve the fiber supply for the pulp and paper industry. Our intent is to use these techniques to alter wood chemistry and fiber characteristics in tree species of commercial importance to the forest products industries of Georgia and the southeastern US, and in the process train new scientists to meet the needs of forestry in the future. Our efforts are currently focused on hardwood trees because techniques to genetically engineer softwood trees on a reproducible basis have not yet been established. However, once such techniques do become available, the researchers we have trained in genetic engineering of hardwood trees should prove equally capable of using them to attack problems inherent to softwood production. To the best of our knowledge, this research program represents the only organized effort to use genetic engineering to specifically improve hardwood species of commercial importance to the southeastern US.

This project was initiated in FY94, and since that time the work at UGA has focused on decreasing the lignin content of yellow-poplar (*Liriodendron tulipifera*) by reducing the expression of laccase, an enzyme which appears to be required for the final steps in lignin biosynthesis. We have isolated the yellow-poplar laccase gene and used microparticle bombardment to introduce it back into yellow-poplar in an 'antisense' orientation in an effort to block laccase synthesis. If the laccase enzyme is truly critical for lignin biosynthesis, the resultant transgenic trees should have a reduced lignin content. Trees with lower lignin content would require less energy to pulp and less chemical treatment to bleach. Our efforts with yellow-poplar in FY96 will focus principally on studies of the pulping and bleaching properties of the transgenic trees currently under production. Somewhat more effort will be focused on sweetgum (*Liquidambar styraciflua*), the hardwood species of greatest economic importance to the pulp and paper industry in Georgia. Depending on the results of studies if FY95, we will use either microparticle bombardment or biological (*Agrobacterium tumefaciens*) transformation to introduce various laccase constructs into sweetgum during the first half of FY96. The resultant transformed cell lines and regenerated trees will be analyzed with respect to lignin deposition, laccase expression, and growth characteristics during the latter half of FY96. During that time, we will also initiate inquiries with appropriate state and federal agencies concerning field tetsing of transgenic trees.

Researchers at IPST have focused on introducing genes affecting auxin metabolism into lines of eastern cottonwood (*Populus deltoides*) which had previously been selected for rapid volume growth. The intent of this work is to find ways to vary auxin levels in plants so as to increase the numbers of fibers and thereby increase yields, or even alter fiber morphology in ways that could lead to improved quality of paper products. A biological transformation system based on *A. tumefaciens* was used to introduce the gene constructs into tissue cultured cottonwood, and Southern blot analysis showed that the gene construct was stably integrated into the cottonwood genome. A new construct carrying the auxin synthase gene under the control of a heat-shock promoter is currently being used in an effort to address initial difficulties in regeneration of trees from the transformed tissues. Efforts at IPST in FY96 will focus on analysis of auxin metabolism and growth characteristics of the regenrated transgenic cottonwood. Special attention will be given to characterizing the properties of fibers recovered from these transformants. Additional collaborative effort between the UGA and IPST groups will focus on the possible use of microparticle bombardment as a machanism for transforming cottonwood tissue lines that have so far proven intractable to transformation by *A. tumefaciens*.

Keywords: tree improvement, genetic engineering, lignin, auxin, cell culture, transformation, regeneration, fiber morphology, "gene gun", *Agrobacterium tumefaciens*

POLLUTION AND WATER DEFICIT-INDUCIBLE GENE EXPRESSION: GLYCINE-RICH PROTEINS

R. J. Newton, A. J. Reinisch, J. Cairney, and R. B. Flagler

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ABSTRACT

The strengthening of the cell wall is one line of defense manifested by plants against external environmental stress. External stress stimuli such as ozone, sulfur dioxide, and water deficit result in the production of oxidative products such as hydrogen peroxide. Furthermore, these same external stimuli induce glycine-rich protein (*grp*) gene induction in the four-winged saltbush shrub, *Atriplex canescens*. It is postulated that hydrogen peroxide functions within the pathway of signal transduction and regulation of nuclear gene expression, resulting in *grp* induction. The inducible *grp* genes are believed to possess promoter sequences which serve as targets for stress-induced transcription factors, and that common, generic signal transduction pathways exist in response to different external stress stimuli. An additional role for hydrogen peroxide is hypothesized to be in its use as a substrate in the cross-linking and insolubilization of GRPs resulting in less elasticity in the cell wall. The research objectives are as follows: (1) Determine exposure concentration/levels and time course of *grp* induction in *Atriplex* tissues, (2) Localize GRPs in cells and tissues, (3) Determine the role of hydrogen peroxide in *grp* expression, GRP solubility and modification of cell wall properties, (4) Determine the effects of prolonged pollution and water deficit stress on tissue elasticity, and (5) Determine the expression pattern of *grp* promoter sequences in transgenic *Atriplex* and transgenic tobacco tissues in response to pollution and water deficit stress.

EPA Environmental Technology Initiative Proposal Cover Sheet

1. Project Title

Pollution Prevention in Paper Mills: Reducing Pulping Waste Through Genetic Engineering of Lignin Synthesis

2. Abstract

To produce paper for printing, lignin, the molecular glue which holds cellulose fibers together, must first be removed from wood chips. The chemical processes used are costly and produce a large amount of by-product waste. Trees of low lignin content would be easier to pulp and would produce less waste. We will use "antisense" technology, to alter the lignin content by gene transfer into plant cells. Both hardwoods and model plants receiving the "antisense" genes will be regenerated as mature transgenic plants and assayed for lignin content. A general reduction and a stem-specific reduction of lignin synthesis will be attempted.

3. Keywords

Lignin, antisense, gene transfer

4. Topic and Major Focus Area

Pollution Prevention Technology. Development and Commercialization of promising new Pollution Prevention Technologies.

5. Total Project Budget $ 1 143 941	**6. Amount Requested from ETI** $ 562 478
7. Submitting Organization and Contact Person U.S. Dairy Forage Research Center USDA-ARS 1925 Linden Drive West Madison, WI 53706 Contact: Dr. John Ralph Tel: 608 - 264-5407 Fax: 608 - 264-5275	**8. Major Partners** Institute of Paper Science and Technology 500 10th Street, N.W. Atlanta, Georgia 30318 Contact: Dr. John Cairney/Tel: 404 - 894-1079 Texas A&M University, Dept. Forest Science College Station, TX 77843 Contact: Dr. Ronald J. Newton Tel: 409 - 845-8279

Shaded Area for EPA Use Only

9. Date Received	**10. Proposal Number**

11. Committee Assignment

12. Comments

Do not include proprietary or confidential information in your proposal. Applications will not be considered unless all requested information is provided.

EPA Environmental Technology Initiative Proposal
FY 95

1. Project Title Enzymatic Deinking of Recycled Office Waste

2. Abstract

This project is directed at pollution prevention by source reduction as well as sludge reduction in recycling processes. Office waste papers contain inks from non-impact printing processes that are difficult to remove with conventional chemical and mechanical processing. Much of this waste is therefore sent to landfill disposal because of the lack of cost-effective technologies to process it. The proposed project will specifically demonstrate on a pilot and mill scale the feasibility of a novel paper recycling process that uses enzymes to facilitate the removal of inks from mixed office waste. An effective process will permit more of this waste stream to be reused and provide recycling mills with the capability to include more post-consumer waste paper in their final product as required by both many consumer groups and government agencies.

3. Keywords Recycled Paper, Mixed Office Waste, Enzymatic Deinking, Sludge Reduction.

4. Topic and Major Focus Area
Topic - Pollution Prevention Focus area - Unit Operations and Functional Areas

5. Total Project Budget $426,880

6. Amount requested from ETI $221,380

7. Submitting Organization and Contact Person

University of Georgia
Dept. of Biochemistry
Athens, GA 30605

Contact: Dr. Karl-Erik L. Eriksson
Phone: 706-542-1686
Fax: 706-542-2222

8. Major Partners

Institute of Paper Science and Technology
500 10th St. NW
Atlanta, GA 30318

Contact: Mr. Charles E. Courchene
Phone: 404-853-9698
Fax: 404-853-9510

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9. Date Received

10. Proposal Number

11. Committee Assignment

12. Comments

PROSPECTUS

FAST-GROWING TREES FOR CARBON RECYCLING, ENERGY AND WOOD PRODUCTION, AND RURAL DEVELOPMENT

BACKGROUND

In recent decades, restrictions on construction of nuclear electric power generating facilities have increased reliance on coal-fired generation. Simultaneously, concern over contribution of carbon dioxide from coal-fired generation to global warming via the greenhouse effect has prompted interest in forest trees as means for sequestering excess carbon and source of renewable fuel.

On a separate but related front, demand for hardwood tree species by the forest products industry has increased beyond expectations. Part of this increase has been caused by pulp and paper companies changing product mixes from commodity to higher value paper grades; the latter require much larger proportions of hardwood fiber. The supply situation, however, has been particularly aggravated in recent times by continuing reductions in availability of timber from lands owned by federal and other governments. As an example, the 70% reduction in harvests from federal lands in the Pacific Northwest has already provoked higher wood prices in the Southeast. Such trends have serious implications for profitability of solid wood and paper manufacturing companies. Tennessee is home to numerous such companies, including primary pulp and paper mills that manufacture products ranging from linerboard and corrugating medium through newsprint to fine printing and writing papers. Daily pulp and paper production exceeds 6500 tons.

An approach to resolving these issues may be found in yet another problem facing our nation, the downward trend in the economic well-being of rural communities. The latest national census indicates that farm numbers continue to decline across the country. Operating cost and product price pressures are forcing consolidation of the best crop lands into fewer and larger farms. More and more rural residents must therefore seek employment off the farm, and significant portions of their often small landholdings go unused or are not used profitably. Imaginative efforts are needed to develop and deploy alternative crops that will raise returns from unused or marginally used lands.

OPPORTUNITY

Developing technologies and genetically improved plant materials for intensively managed plantations of fast-growing trees provides an opportune means for meeting these congruent needs. Such plantations would provide for increasing and accelerating carbon fixation and make available a potential supply of renewable fuel

PROJECT SUMMARY

The Project Summary should include a statement of objectives, methods to be employed, and the significance of the proposed activity to the advancement of knowledge or education. Avoid use of first person to complete this summary. **DO NOT EXCEED ONE PAGE.** (Some Programs may impose more stringent limits.)

Acquisition of Biotechnology Equipment for Forest Tree Improvement

The U.S. Forest Products Industry is a major force in the U.S. economy comprising approximately 7% of the U.S. manufacturing output and employing some 1.4 million people with a $46 billion annual payroll and annual sales approaching $200 billion. Currently, much of this U.S. industry is faced with an uncertain future because of the reduced availability of the timber resources that supply the industry's basic raw material needs. This shortage results from a loss of growth due to environmental stress, pathogens and pests, a loss of harvestable forest land due to environmental restrictions, and a loss of productive forest lands to urban growth and development. This loss in tree growth and land is compounded by availability of cheap foreign fiber supplies from rapidly growing tropical trees. If the productivity of U.S. forests cannot be increased within the next decade it is likely that much of the industry will turn to importation of raw materials or relocation. This lends urgency to research efforts to boost forest productivity domestically and to protect forests from the damaging effects of environmental stresses.

A potential solution to this problem is offered by the modern techniques of molecular biology. The molecular biology tools available today offer the potential to intensively study the many physiological mechanisms responsible for forest tree growth, environmental stress tolerance, and pest resistance and tolerance. Through the use of these tools and the knowledge generated, plants may be genetically altered for improved survival and growth rates. Clonal propagation of high value genetically altered fast-growing trees, in a crop-like setting, offers the potential of meeting future U.S. needs for high quality forest raw materials.

Biotechnology will play an important role in the agricultural and forest industries of the future. Much agricultural research now focuses on biotechnology. However, few forest biology laboratories are equipped with the instrumentation required for the complex creation, evaluation, and storage of genetically altered forest trees. By exploiting recent advances in biotechnology and applying these to forest tree research, U.S. universities can reaffirm their leadership role in research and teaching while U.S. industries maintain their competitive edge. To realize this potential there must exist strong communicative links between academia and industry, and the technical resources must be available to carry out the appropriate research. The unique position of IPST places it at a communication interface; therefore, this proposal applies for funds to acquire equipment for the genetic modification, growth, evaluation, and long-term storage of transformed forest trees.

Requested Equipment:

1. Cryogenic storage equipment is required for the long-term storage of genetically altered and non-altered coniferous plant cultures in the form of early-stage somatic embryos.
2. State of the art "projectile particle delivery " and electroporation equipment is needed for the efficient transformation of forest tree tissue, and bacteria , including *Agrobacterium tumefaciens*.
3. A state-of-the-art autoclave is needed to precisely control temperature and pressure-induced breakdown of media components which impact media osmolality and embryo development.
4. Three growth chambers are needed to produce specific "stress" environments and for the conversion of somatic embryos into autotrophic forest tree seedlings.
5. Light detection equipment is needed for use of a Luciferase gene transfer assay.
6. A oligonucleotide synthesizer , spectrophotometer, and laminar flow transfer hood are requested for the routine sequencing, assays, and sterile transfers associated with tissue culture & genetic engineering work.

Activities that will result from procurement and use of requested equipment:

1. High technology research focused on growth rate improvement of forest trees.
2. Creation of a high-ranking, state-of-the-art forest tree-oriented molecular biology center ; the only plant-related molecular biology center in Northern Georgia.
3. State-of-the-art research oriented to improvement of pulp & paper and specific forest tree products.
4. Training of biotechnologists in state-of-the-art laboratories focused on forest tree growth and products.
5. Increased collaboration and bridging of biotechnology programs at IPST, universities and private industry.
6. Involvement of and interaction between IPST and minority institution biology programs.

Impact of use of requested equipment:

1. Creation of trees with improved growth rates that will keep U.S. Industry competitive.
2. Develop new jobs in a forest biotechnology industry.
3. Train scientists to apply molecular biology to forest trees.

Publications

PUBLICATIONS - 1994-1995
(Issued, in press, or submitted)

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Student Research

STUDENT RESEARCH - COMPREHENSIVE LIST

Following is a list of students in the Forest Biology Group along with their project or thesis titles and a summary of the work proposed or in progress. Projects with a * are specifically targeted at the Softwoods Project.

Yi Ren Chen (M. Sc.)
Title: Enzymatic Deinking and Washing
Advisor: Ron Dinus / Peter Pfromm
Summary: Waste paper recycling has increased in recent years, and is likely to continue rising over the foreseeable future. New technologies, e.g., enzymatic deinking, for improved ink removal would enhance prospects for using even larger quantities of waste paper. The enzymatic deinking process utilizes enzymes, primarily cellulases and hemicellulases, to dislodge ink particles from fiber surfaces. Ink particles released by enzymatic action are then removed from fiber suspensions by washing and/or flotation. A first goal of this project concerns optimal conditions for action of selected enzymes. Several factors must be considered, including enzyme type, enzyme concentration, reaction time, temperature, pH, pulp consistency, and surfactant concentration. A second goal involves gauging effectiveness of enzymatic deinking. Washing seems an ideal method for removing maximum quantities of ink dislodged by enzymatic or conventional deinking. Extensive washing will therefore bye used to remove ink, with brightness tests and ink particle counts via image analysis used to quantify ink left after enzymatic and conventional deinking. The research should yield valuable and original data on the quality of ink removed by enzymatic treatments as compared to that freed by conventional chemical deinking.

Vincent Ciavatta (M. Sc.) *
Title: Development of an assay method for measuring indole-3-acetic acid in loblolly pine embryos.
Advisor: Ron Dinus
Summary: Knowledge of the ebb and flow of plant hormones in developing loblolly pin (*Pinus taeda*) zygotic embryos is important in establishing a model for somatic embryogenesis of loblolly pine. To this end, abscisic acid has been quantified in loblolly pine zygotic embryos through an indirect ELISA (enzyme-linked immunosorbent assay) (Kapik et al., 1993). The goal of my work is to quantify indole-3-acetic acid (IAA) in developing loblolly pine zygotic embryos via a novel direct ELISA. In addition to providing information useful to the loblolly pine somatic embryogenesis effort, the development of this ELISA will provide a quick and efficient method for measuring minute quantities of IAA in many other plant tissues.

Tim Crocker (M. Sc.)
Title: Lignin and Holo-Cellulose Contents of *Pinus taeda* Progenies using a FTIR Analytical Technique.

Advisor: Ron Dinus

Summary: The Lignin and Holo-Cellulose content of increment cores from selected families of *Pinus taeda* will be determined using an FTIR diffuse reflectance technique. The Lignin and Holo-Cellulose data will be used to determine variation within families, variation among families, and a calculation of heritability of Lignin and Holo-Cellulose contents maybe attempted. This may also provide the opportunity to examine correlations of Lignin and Holo-Cellulose contents and other traits; for example specific gravity. Sampled families will be offspring from parents that have documented superior growth characteristics. The advantage of the FTIR technique over classical wet methods is realized by non-destructive sampling, small sample size, easy sample preparation, and rapid execution.

Brian Klunk (M. Sc.) *

Title: Reduction of Lignin Content via Genetic Improvement Methods

Advisor: John Cairney

Summary: Very large contributions to both the cost of producing marketable pulp and the pollutants created during the process stem from the removal of lignin. Genetic methods have demonstrated that both the type and quantity of lignin present in wood can be altered. Previous attempts to reduce lignin content have resulted in general reduction of lignin throughout the plant. This reduction compromises the trees natural ability to defend itself against biological attacks. Isolation of lignin synthesis genes and promoters which operate solely in the stem and are not induced by environmental stresses is the primary goal of my work. Starting with a cDNA for *O*-methyltransferase found in loblolly pine by Cairney, *et al* I will be constructing a cDNA library from which to isolate the genomic clones of this gene. Once these clones are isolated, the RAGE (random amplification of genomic ends) method will be used to isolate their promoters. It will then be possible to clone these promoters into the promoterless GUS vector for subsequent location of gene activity in transgenic plants.

Tom Kraker (M. Sc.) *

Title: Identification of stem-specific genes in loblolly pine using differential display.

Advisor: John Cairney

Summary: "Antisense" technology, whereby a reverse-orientation gene fragment is transferred into plants, is capable of turning down or turning off specific genes. This method has great potential for reducing lignin content of trees through transfer of "antisense" lignin synthesis gene fragments into particular trees. However, lignin synthesis is known to be a defense response in plants. If the amount of lignin is non-specifically reduced by genetic engineering, disease-susceptible plants may result. This problem will be avoided if lignin reduction is confined to the stems in a fashion that does not compromise the defense capability of the plant. Stem-specific expression of an antisense lignin synthesis enzyme using a stem-specific promoter which is not involved in defense response would achieve this goal; however, few stem-specific genes have been identified. The technique of Differential Display will be used to identify stem-specific genes. cDNA copies of these genes will be cloned and sequenced. Subsequently, stem-

specific promoters may be isolated. Insertion of "antisense" lignin o-methyltransferase cDNA into commercially important tree species in antisense orientation with stem-specific promoters will allow production of trees with less structural lignin, but whose defense capabilities are still intact. This would significantly reduce the costs and wastes involved with conventional chemical pulping.

Argentina Leyva (M. Sc.) *

Title:

Advisors Jerry Pullman, Don Dimmel, John Cairney

Summary: Genetic improvement of forest trees to increase the amount of naturally occurring anthraquinones which could be used as catalysts in pulping.

Summary: Anthraquinones (AQ) are known to increase wood pulping yields by acting as pulping catalysts. The high cost of AQ provides the reason to investigate the possibility of producing AQ naturally in the pulping trees themselves. Excellent AQ derivatives have been found to be produced in plants. Some of these derivatives are effective as pulping catalysts but are produced either in non-pulping species or in very small amounts. The most promising finding from the literature is that Teakwood (Tectona grandis) contains 0.1-0.5% 2-methyl AQ (tectoquinone), approximately 2-10x the concentrations needed for pulping loblolly pine. Tectoquinone is highly rated as an AQ for pulping efficiency. This information suggests that genes for AQ production may be isolated and transferred to forest trees of commercial interest. The goals of my A190 are to isolate and quantify tectoquinone from teakwood and compare the pulping effectiveness of industrial vs. natural tectoquinone. Pulping tests with loblolly pine will be developed to determine kappa number, lignin removal curves, and yield for varying concentrations of Teakwood, isolated AQ, or industrial AQ. A long range view of this project is to build information which increases the understanding of pathways involved in the production of AQ in plants, the identification and isolation of genes responsible for production of AQ in plants, and the eventual movement of genes into target pulping species.

Andy Toering (M. Sc.) *

Title: The Development of a Mathematical Rate Adsorption Equation Derived Explicitly for the Adsorption of 2,4-dichlorophenoxyacetic acid by Activated Carbon in Tissue Culture Media.

Advisor: Jerry Pullman

Summary: The focus of my research centers around the special sorption properties of activated carbon, with particular emphasis placed on the ability of the activated carbon to adsorb plant growth regulators in tissue culture media. It has been shown that the addition of activated carbon to tissue culture media helps promote the growth of cells. We believe that this is in part due to the sorption of these growth regulators on activated carbon which occurs at a reproducible rate. Our goal is to quantify this rate based on variable amounts of activated carbon and plant growth regulators, and to develop a dependable model from this information. To do so, I will be using C^{14} labeled 2,4-dichlorophenoxyacetic acid, and observing it's rate of disappearance from the media as it is being sorbed onto the carbon.

Drake Walsh ((M. Sc.) *

Title: In preparation

Advisors: Jerry Pullman / David Rothbard

Summary: I am currently a full-time IPST employee in the Research Services Division Fiber Microscopy Group. I am also a part-time IPST Masters student. I am currently beginning my A190 research proposal and plan to observe zygotic and somatic embryos of loblolly pine with the scanning and transmission electron microscopes. The focus of my project will be on defining the similarities and differences between zygotic and somatic embryos with a focus on early stages. This work will include sample preparation, morphological characteristics and x-ray analysis for potential identification of elemental composition. First, the scanning electron microscope will be used because of its use in surface characteristics and depth of field. Later, I plan to embed embryos in epoxy, take ultra thin sections, and perform observational as well as elemental mapping.

Lois Ford Kohler (Ph. D.)

Title: The Effects of *Ophiostoma piliferum* on Wood Pulp: Investigating the Impact of Ophiostoma piliferum on the Strength Properties of Handsheets

Advisor: Ron Dinus

Summary: Efforts centered on measuring, via conductometric titration, numbers of acid groups in pulps treated and not treated with Ophiostoma piliferum. Various exposure times were also tested. Preliminary results indicate significant differences among treatments, with pulps exposed to O. piliferum having larger numbers of acid groups. Efforts to retest these and related experiments were also initiated. Approximately 1.5 tons of loblolly pine chips were secured from Bowaters Corp., Calhoun, TN; half were frozen for use as controls and half were inoculated under controlled conditions with O. piliferum. Following five weeks of exposure, treated chips will be frozen and shipped, along with control chips, to Andritz-Sprout-Bauer Company, Springfield, OH for refining. Pulps will be recovered and tested as usual. Papers are being prepared for presentation at the International conference on biotechnology in the pulp and paper industry scheduled for this June in Vienna, Austria, and at the TAPPI pulping conference scheduled for October in Chicago.

Rene Kapik (Ph. D., Matriculates in June, 1995) *

Title: A study of plant growth regulators during zygotic embryogenesis in loblolly pine.

Advisor: Ron Dinus

Summary: An abstract of her research is presented at the end of this section.

Deborah Villalon (Ph. D.) *

Title: Characterization of a Proteinase Inhibitor from *Atriplex canescens*

Advisor: John Cairney

Summary: The main objective of this project is to study mechanisms of plant response to environmental stress at the molecular level. The ultimate goal is to express stress resistance genes in loblolly pine using the techniques of molecular biology and

plant tissue culture to develop a genetically superior tree. A gene family of drought inducible cDNA clones that code for a Proteinase Inhibitor (PI) have been isolated from the woody desert shrub, *Atriplex canescens*. Two corresponding genomic clones have been isolated. Further characterization at the level of gene transcription and steady state RNA is being conducted using total RNA extracted from drought stressed plants. The question of gene function is being addressed by using an *in vitro* bacterial gene fusion expression system. Protein purified in this method can be subjected to a PI radial inhibitor diffusion assay. To identify sequences thought to be involved with gene regulation , the commonly used β-glucoronidase (GUS) reporter gene system has been chosen. The polymerase chain reaction (PCR) amplification of promoter and terminator regions of the genomic clones has been used to construct recombinant plasmids containing the GUS gene. These constructs will be introduced into *Agrobacterium tumefaciens* using cell electroporation. Genetically altered strains of *A. tumefaciens* will be used to introduce and express these recombinant genes in the host plant, *Arabidopsis thaliana.* The techniques for plant regeneration and transformation using this model system have been established in this lab.

Stephen Van Winkle (Ph. D.) *
Title: An investigation into an unsuccessful tissue culture medium: Determining the role of activated charcoal.
Advisor: Jerry Pullman
Summary: Previous tissue culture experiments with two different charcoals supplied by Sigma revealed that one charcoal promoted embryogenesis of Douglas-fir while the other did not. The goal of this project is to discover why one charcoal was ineffective. Research will be directed towards physically and chemically characterizing many (~20+) different charcoals with the goal of correlating these characteristics with charcoal performance in tissue culture medium. Charcoal is known to be a versatile sorbent: performance will be measured in terms of sorption of tissue culture medium components (particularly hormone and mineral nutrients). Performance will also be measured using a bioassay model for the Douglas-fir initiation system. This bioassay will be developed using Norway Spruce zygotic and somatic embryos. Initial work will focus on the two Sigma charcoals and bioassay development.

Colleen Walker (Ph. D., Matriculates in June, 1995)
Title: Selectivity of iron-based catalysts in a polymeric model system for biomimetic bleaching. (Colleen defended and completed her thesis.)
Advisor: Ron Dinus
Summary: An abstract of her research is presented at the end of this section.

Thomas Welt (Ph. D.)
Title: Enzymatic deinking - Effectiveness and mechanisms.
Advisor: Ron Dinus
Summary: Although several theories explaining enzymatic deinking have been proposed few studies have focused on the mechanism(s) involved. Therefore, the overall

objective of the present study is to generate data which will yield a better understanding of the mechanisms involved in enzymatic deinking. More specifically we will evaluate the effect of enzymatic action on ink and fibers, and how these actions affect ink release during paper disintegration. A well-defined paper material and highly purified enzymes will be used throughout the study. A technique for visualization of the enzymatic attack on pulp fibers will be developed. Scanning electron microscopy (SEM) will be used to study surface changes caused by enzymatic action. Colloidal gold coated antibodies directed against enzymes or gold-labeled enzymes and transmission electron microscopy (TEM) will be employed to help visualize enzymatic attack on and inside fiber walls. In addition, research efforts will focus on methods to immobilize enzymes. These methods will help to determine: 1) individual effects of a particular enzyme type; 2) if synergistic effects between enzymes are important in deinking; 3) the spatial distribution of enzymatic attack; and 4) the effect of surface and/or internal action of enzymes on pulp fibers.

Mike Wood (Ph. D.) *

Title: Examination of genetic structure in *Pinus elliottii* populations using the polymerase chain reaction.

Advisor: Ron Dinus

Summary: Practices used to manage populations of trees may alter their genetic makeup. Some practices actively influence genetic structure, while others inadvertently produce change, e.g., selection and drift. Understanding the magnitude and direction of these changes may allow for improved decisions concerning future generations. The objectives of this work include, demonstrating the ability to quantify genetic structure in individuals and populations of slash pine, and use of this ability to investigate issues concerning a tree improvement program. These issues include, measuring the amount and distribution of variation within populations, and the effect of management practices on its distribution.

CHANGES IN ABSCISIC ACID CONCENTRATION DURING ZYGOTIC EMBRYOGENESIS IN LOBLOLLY PINE (*PINUS TAEDA*) AS DETERMINED BY INDIRECT ELISA

A Dissertation Submitted by

René Howard Kapik

B. S. 1984, Western Michigan University

M. S. 1986, Lawrence University (Institute of Paper Chemistry)

In partial fulfillment of the requirements
for the degree of Doctor of Philosophy
from The Institute of Paper Science and Technology
Atlanta, Georgia

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ABSTRACT

No studies published to date have documented changes in endogenous ABA levels in zygotic conifer tissues. This research examined fluctuations of endogenous (+)-abscisic acid (ABA) in embryos, megagametophytes, and suspensors during zygotic embryogenesis in loblolly pine (*Pinus taeda*). Methods were developed to collect and store seed tissues, to extract and purify ABA, and to quantitate ABA with an amplified indirect enzyme-linked immunosorbant assay (ELISA). The indirect ELISA, developed with a commercially available monoclonal antibody and amplified using an avidin-biotin-multiple horseradish peroxidase, provided reliable and sensitive (~2 pg/100 µL) estimates for ABA in loblolly pine zygotic tissues. Validation of early, mid, and late zygotic tissues using definitive gas chromatograph/mass spectroscopy (GC/MS) ruled out the presence of interfering substances.

Commonly used methods used were found to influence the final ABA estimates. Separation of embryo, suspensor, and megagametophyte tissues during dissection significantly increased (~50%) the endogenous ABA levels. ABA levels were also increased (~28%) during tissue storage prior to analysis despite stringent storage conditions. Therefore, absolute ABA estimates must be considered carefully, although the overall trends should be accurate.

Results indicated two significant peaks in endogenous ABA levels (dry-weight basis) in the whole ovule. The first major peak occurred early in development and was attributable to megagametophytic ABA. The second peak occurred at mid-development and was attributable to embryonic ABA. Similar to previously documented trends in cereals, legumes, and woody angiosperms, embryonic ABA (dry-weight and per embryo basis) was

extremely low early in development, significantly higher during mid-development, and then rapidly declined to low levels at seed maturity. This trend was found to be similar in four mother trees during the 1992 growing season and across two growing seasons (1992 and 1993) in a single mother tree. Such findings suggest that environmental factors (such as day length) that are common within the geographic regions are critical in regulating endogenous ABA levels. Moreover, it appears that within-species genetic variations play a minor role.

Several causal relationships can be inferred from endogenous ABA levels and physiological changes in loblolly pine zygotic tissues. First, embryonic ABA increased prior to and concurrent with the accumulation of dry-weight in the embryo. This supports the hypothesis that high levels of embryonic ABA may initiate the production of storage products, but are not necessarily required for their continued synthesis. Second, megagametophytic ABA levels and the percent moisture of the megagametophyte and embryo all dropped rapidly after fertilization. This suggests that megagametophytic ABA may initiate and control the rate of desiccation in both tissues. Third, embryonic ABA (μM basis) increased rapidly during late development and increased to an extremely high level at complete seed dormancy. This suggests that a high level of embryonic ABA may be correlated with the suppression of precocious germination as well as induction and maintenance of dormancy in embryos of loblolly pine.

SELECTIVITY OF IRON-BASED CATALYSTS IN A POLYMERIC MODEL SYSTEM FOR BIOMIMETIC BLEACHING

A Dissertation Submitted by

Colleen C. Walker

B. S. 1987, University of Delaware

M. S. 1989, Lawrence University

in partial fulfillment of the requirements
from the Institute of Paper Science and Technology
for the degree of Doctor of Philosophy
Atlanta, Georgia

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September 1994

ABSTRACT

Discovery and characterization of fungal enzymes capable of degrading lignin have suggested the study of simpler compounds to mimic these enzymes. Use of these so-called biomimetic compounds has been extended to applications in bleaching wood pulp. Attempts to bleach pulp with biomimetic compounds have so far failed to demonstrate that these compounds are selective catalysts for pulp delignification.

To be feasible as bleaching agents, such compounds must be selective, i.e. demonstrate high reactivity toward lignin without severely damaging carbohydrates. The goal of this thesis was to evaluate the selectivity of three biomimetic systems. The systems investigated were ferrous sulfate, ferrous ion chelated with ethylenediaminetetraacetic acid (EDTA), and hemoglobin, all in the presence of hydrogen peroxide. A polymeric, homogeneous model system has been used to represent wood pulp. Lignosulfonate and hydroxyethyl cellulose (HEC) were chosen as water-soluble, polymeric models for lignin and carbohydrate. Molecular weight changes of these substrates were measured by High Performance Size-Exclusion Chromatography (HPSEC) and viscometry, respectively.

When these substrates were individually exposed to each biomimetic compound in the presence of hydrogen peroxide, substantial degradation of both lignin and carbohydrate model compounds was observed. Rates of lignosulfonate and HEC degradation were separately determined and compared for each biomimetic catalyst. Hemoglobin was found to be the most selective for lignosulfonate degradation over HEC degradation. In addition, hemoglobin was found to be the most effective for catalyzing the oxidation of lignosulfonate versus decomposition of hydrogen peroxide to oxygen.

During these reactions the production of hydroxyl radicals was measured using a chemiluminescence assay. Although hemoglobin interfered with this assay, results from the

ferrous sulfate and chelated ferrous catalyzed reactions were obtained. Rates of hydroxyl radical production in the ferrous system were directly related to bond cleavage of lignosulfonate.

When lignosulfonate and HEC were simultaneous exposed to hydrogen peroxide and these catalysts, a high molecular weight product was formed. This product is the result of condensation reactions between the lignin and cellulose models. Its formation is significant as it represents a counterproductive process that may be responsible for the limited effectiveness of biomimetic delignification systems.