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FOREST BIOLOGY PROJECT ADVISORY COMMITTEE MEETING

October 18-19, 1994

Institute of Paper Science and Technology
Atlanta, Georgia

TABLE OF CONTENTS

Agenda i

Committee List ii

SOFTWOODS 1

Mass Clonal Propagation of Improved Conifers
Technical Program Review 2

- 1994-95 Milestones 4
- Initiation 9
- Growth Kinetics of Loblolly Pine Early-Stage Embryo Suspensions 23
- Germination 33
- Molecular Biology 36

HARDWOODS 38

Mass Clonal Propagation of Genetically Improved and Engineered Hardwoods
Technical Program Review 39

Research Proposals 43

Publications 54

Student Research 57

FOREST BIOLOGY
ANNUAL RESEARCH REVIEW
AGENDA

October 18, 1994

Time	Item	Presenter
2:00 P.M.	Welcome, introduction, antitrust statement	Malcolm
2:05	Hardwoods - Cottonwood Transformation	Dinus, Stephens
2:45	Molecular Biology - Softwoods & Hardwoods	
	Introduction to Research Projects	Cairney, Chang, Villalon
3:30	Break	
3:45	Softwood Embryogenesis	
	Initiation	Johnson
	Growth Kinetics	Johns
	Germination	Pullman
	Molecular Biology	Cairney

October 19, 1994

Time	Item	Presenter
8:00	Coffee & Donuts	
	Forestry Initiative Updates	
8:30	Proposal Development	Malcolm
	IPST Board of Trustees Subcommittee	Malcolm
9:30	Program Integration Committee	Canavera
10:00	Government Affairs Committee	Coleman
10:15	Technical Program Committee	Leach
10:30	IPST Forest Biology Hardwoods Subcommittee	Lazar
11:15	Future Plans for IPST Research	
	1 Year Plan	
	5 Year Plan	
12:00	Lunch	
1:00	Continued Discussion of Future Plans	

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SOFTWOODS

TECHNICAL PROGRAM REVIEW
FY 94-95

Project Title:	MASS CLONAL PROPAGATION OF IMPROVED CONIFERS
Project Code:	SFTWD
Project Number:	3223-00 (F-010)
Division:	Chemical and Biological Sciences
Project Staff:	Gerald Pullman, John Cairney, Shujun Chang, Barbara Johns, Shannon Johnson, Yolanda Powell, Camille Stephens

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 to form plants in vitro with both shoot and root growth. Somatic embryos from two additional genotypes have germinated to produce a few plants with shoot growth but no root growth yet.

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.

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 to 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.

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.

Six research grant proposals (see the list at the end of this PAC Report), representing over $500,000 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

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.

<u>ANALYSIS</u> 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.

ZYGOTIC INITIATION After summer 1994 cone collections started arriving, at least one somatic embryo genotype continued to be incorporated into the initiation experiments. This allows conclusions to be drawn on the basis of extrusion and initiation as well as continued growth of somatic embryos. Several zygotic initiation experiments are now in progress. Some initiation and growth of the first somatic embryos has been seen but experiments have not reached the point to where they can be fully evaluated.

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.

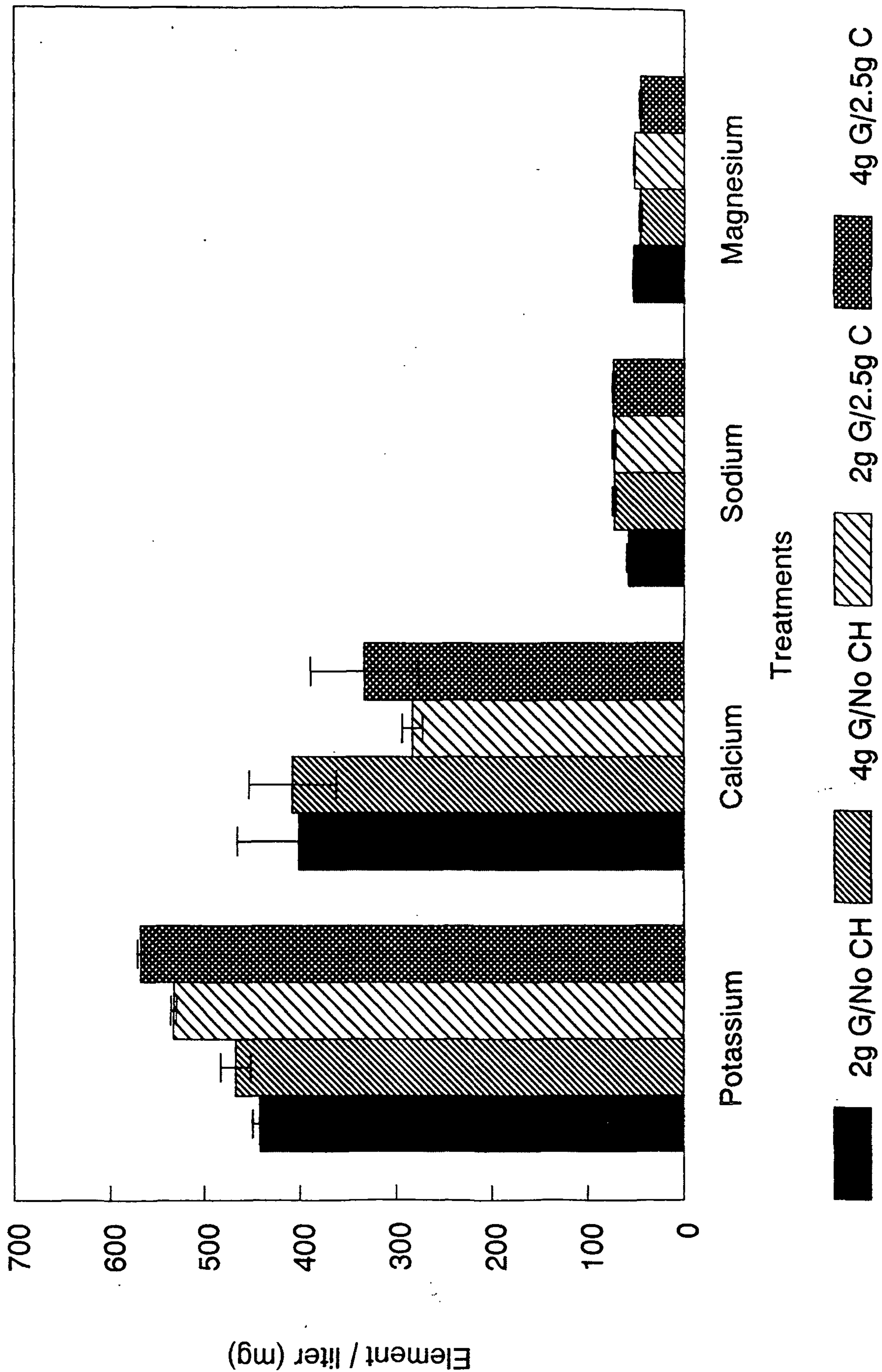


Figure 1. Effect of two gelrite and two charcoal concentrations on the amount (mg/l) of macronutrients (K, Ca, Na and Mg) available in initiation media.

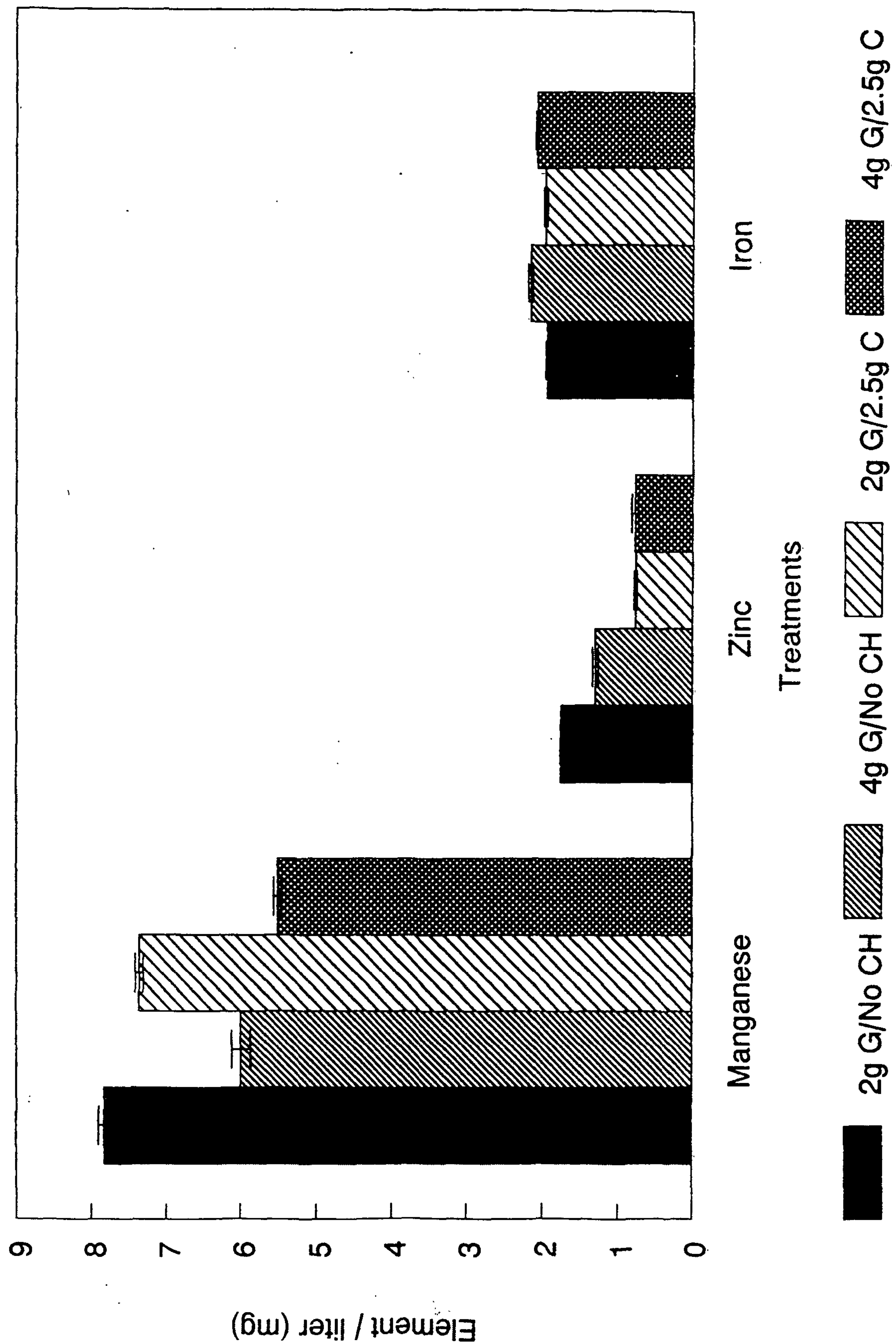


Figure 2. Effect of two gelrite and two charcoal concentrations on the amount (mg/l) of micronutrients (Mn, Zn and Fe) available in initiation media. Note the 50% reduction in the amount of Zn when charcoal is added.

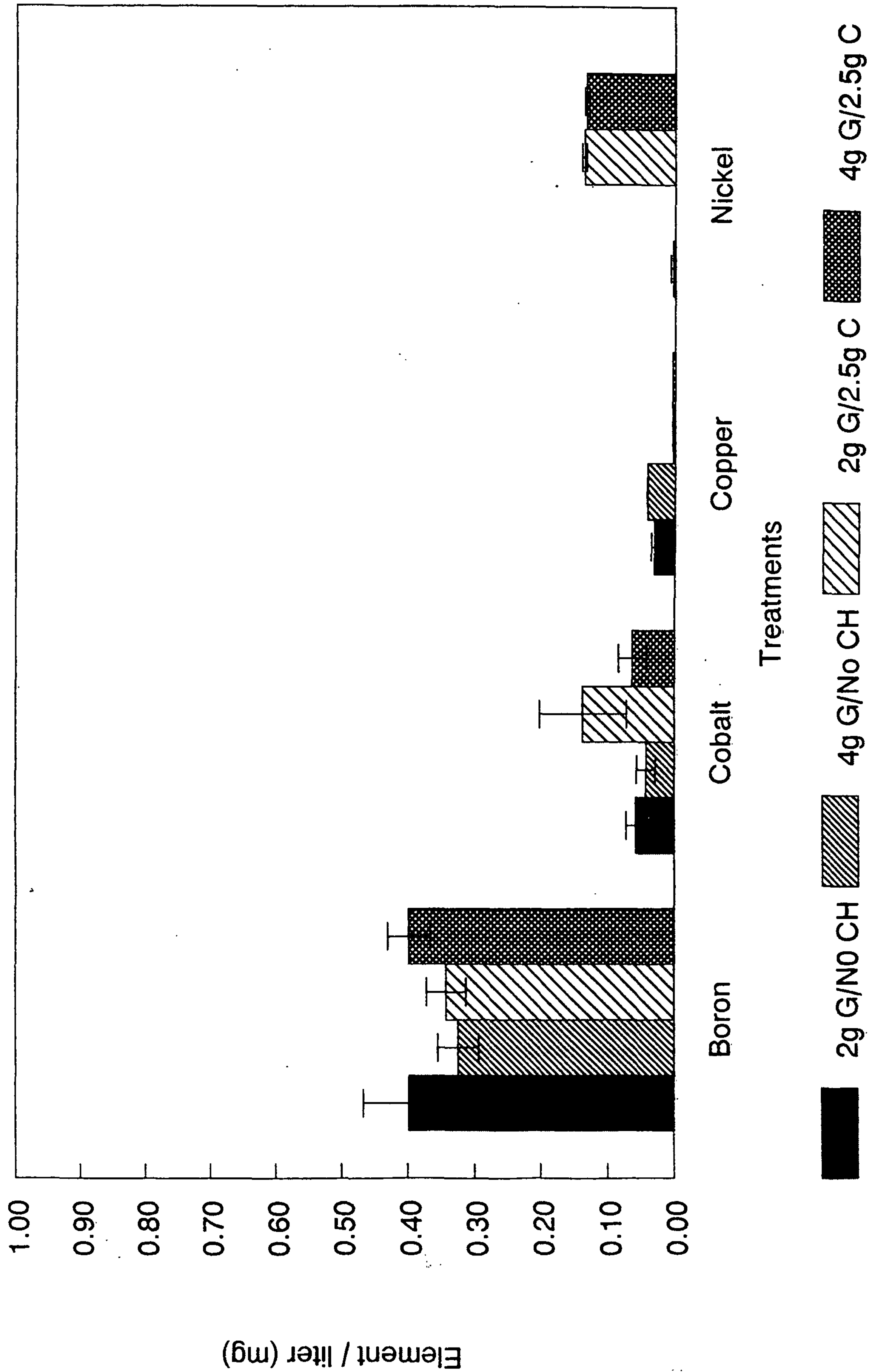


Figure 3. Effect of two gelrite and two charcoal concentrations on the amount (mg/l) of micronutrients (B, Co, Cu and Ni) available in initiation media. Note the 90% reduction in the amount of copper when charcoal is added.

	Gelrite			
Element	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%.

	Charcoal			
Element	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.

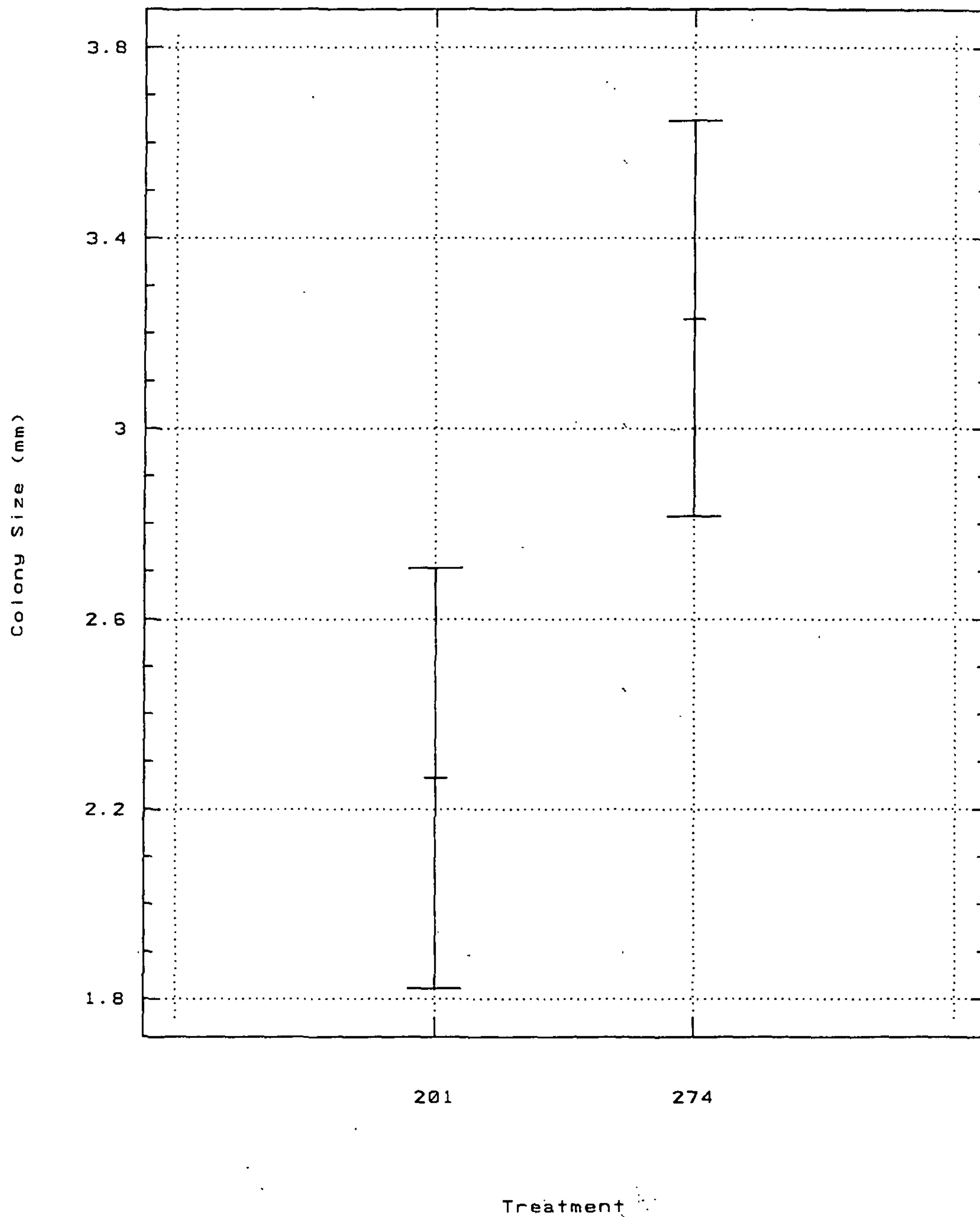


Figure 4. Confidence intervals for basal medium 201 and medium 274 (201 with double the micronutrients). A statistically significant increase in colony size is shown with double the micronutrients treatment.

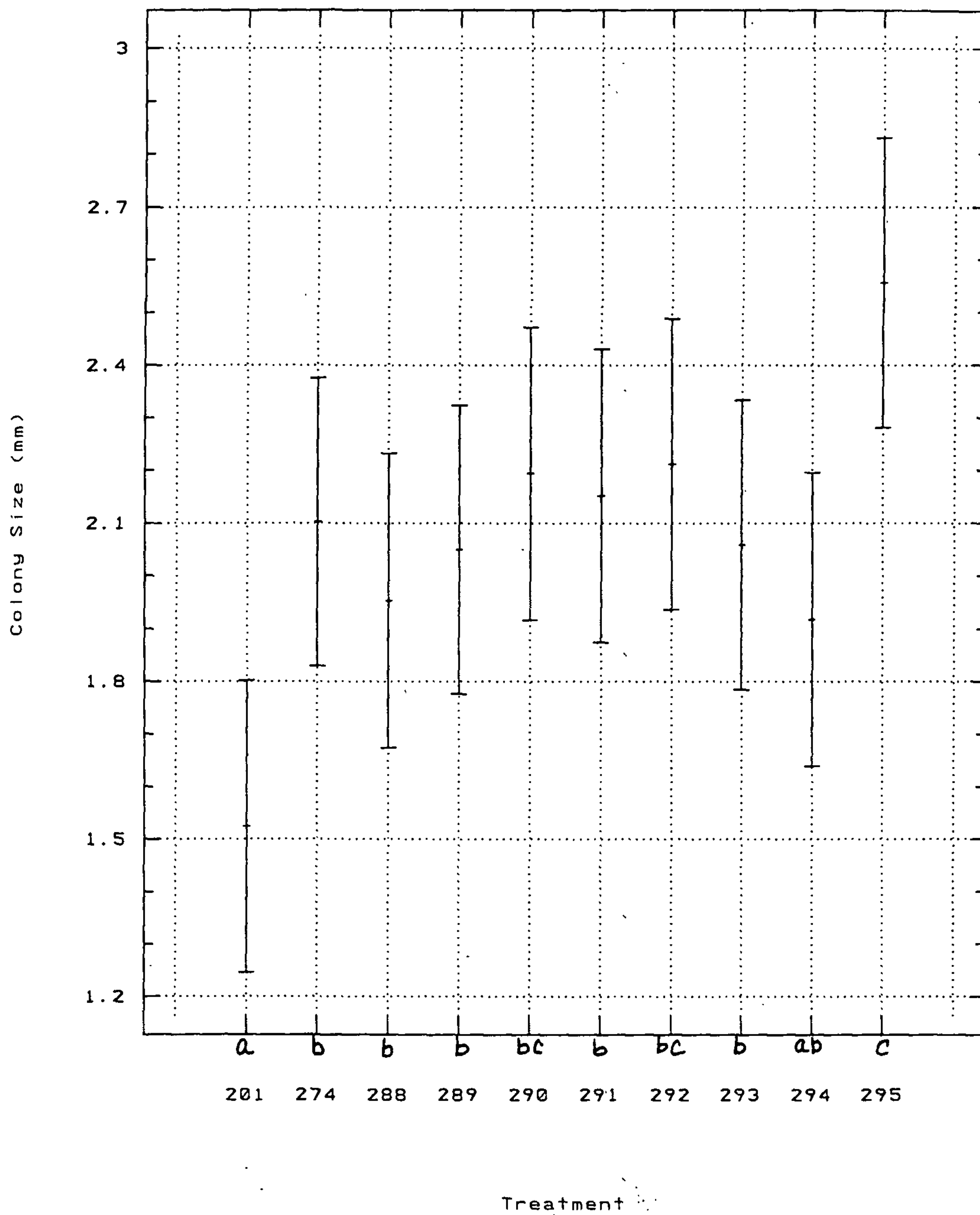


Figure 5. Confidence interval for basal medium 201, double micronutrients medium 274 and a control, without charcoal medium 295. Media 288 - 294 represent treatments with 3X micronutrients or the addition of a single micronutrient. Treatments with the same letter are not significantly different (Duncan's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

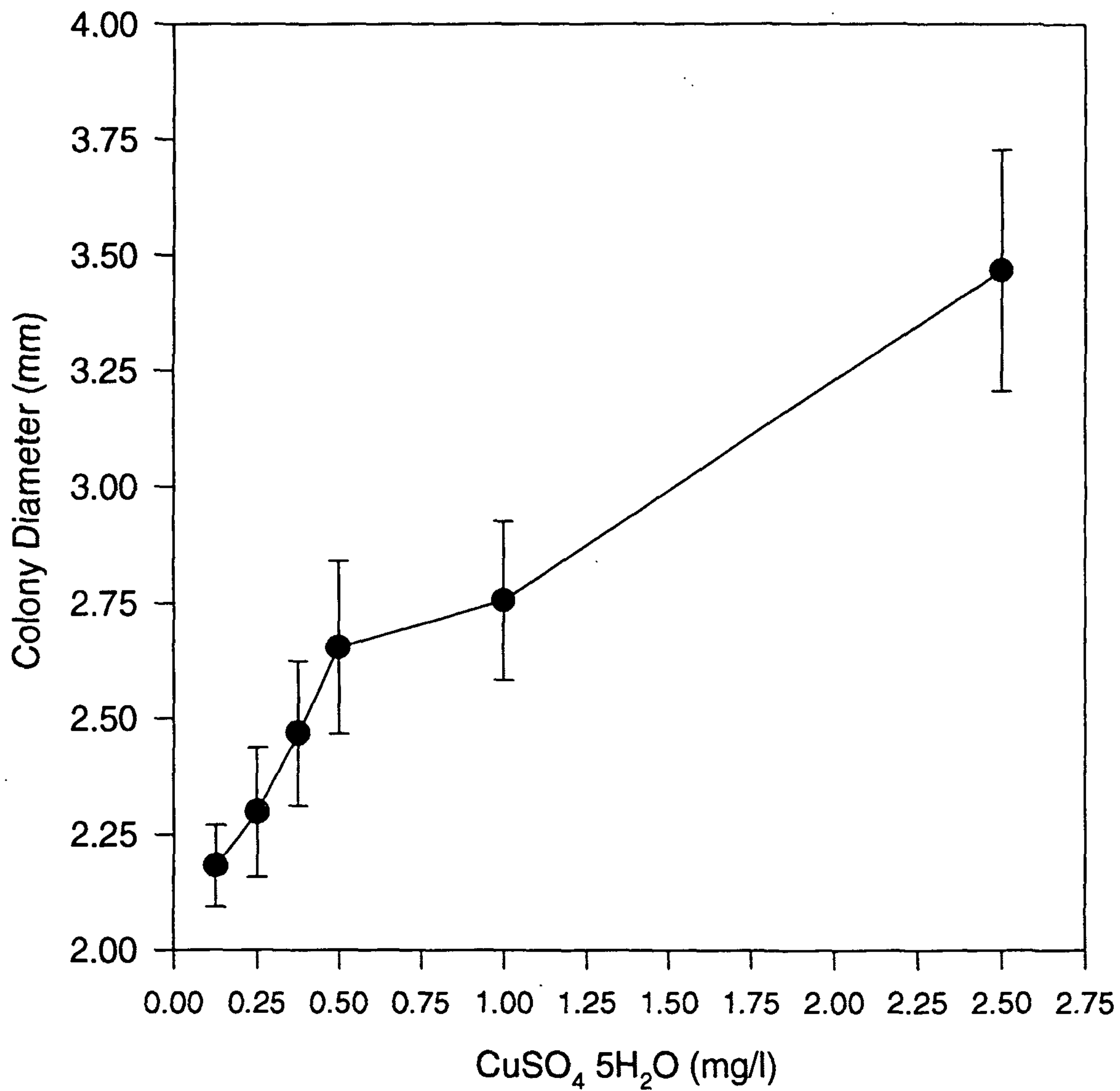


Figure 6. Effect of initial $CuSO_4$ $5H_2O$ concentration in initiation media on the colony diameter of somatic embryos.

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.

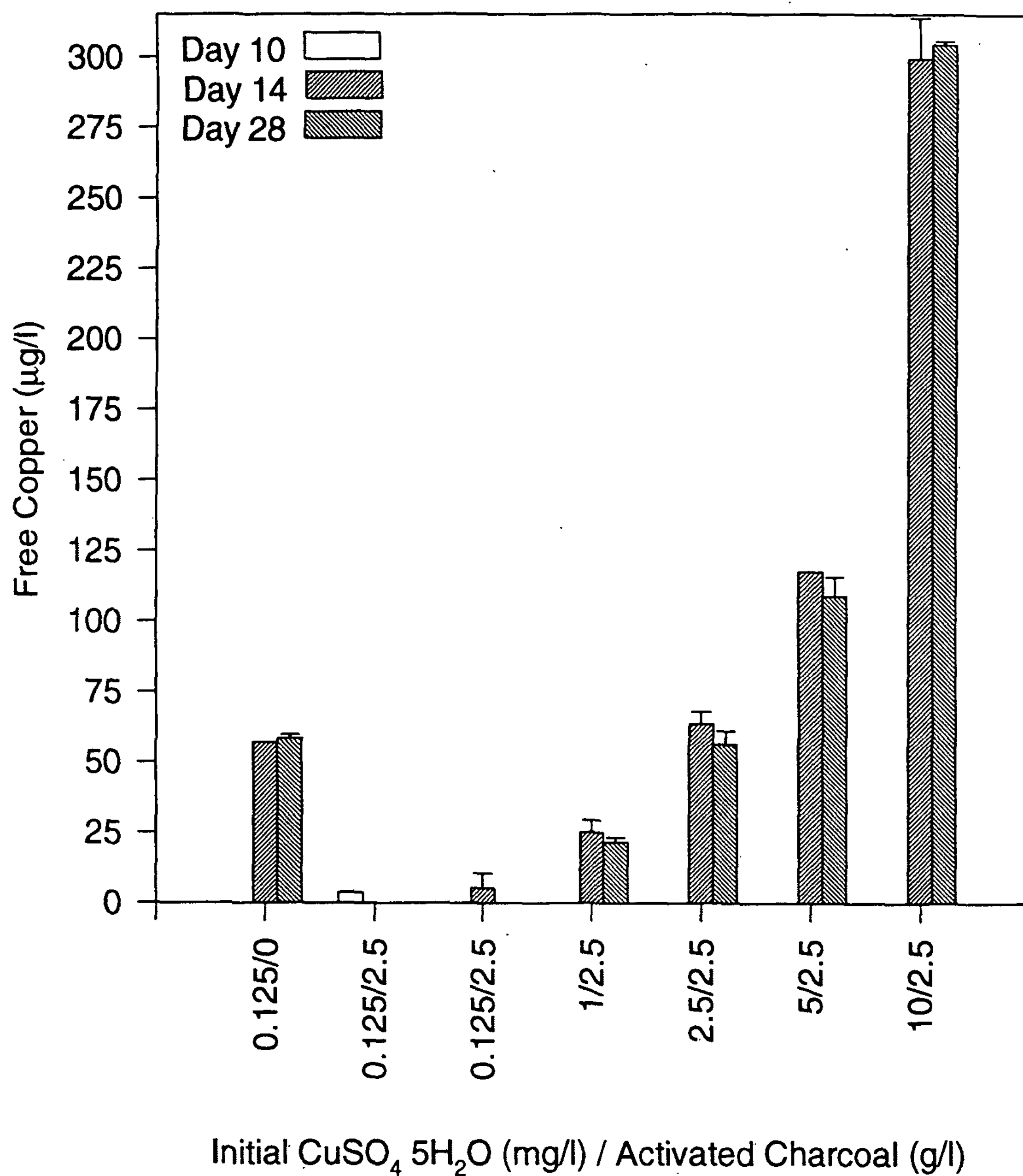


Figure 7. Effect of five $CuSO_4$ $5H_2O$ and two charcoal concentrations on the amount (µg/l) of free Cu available in initiation media.

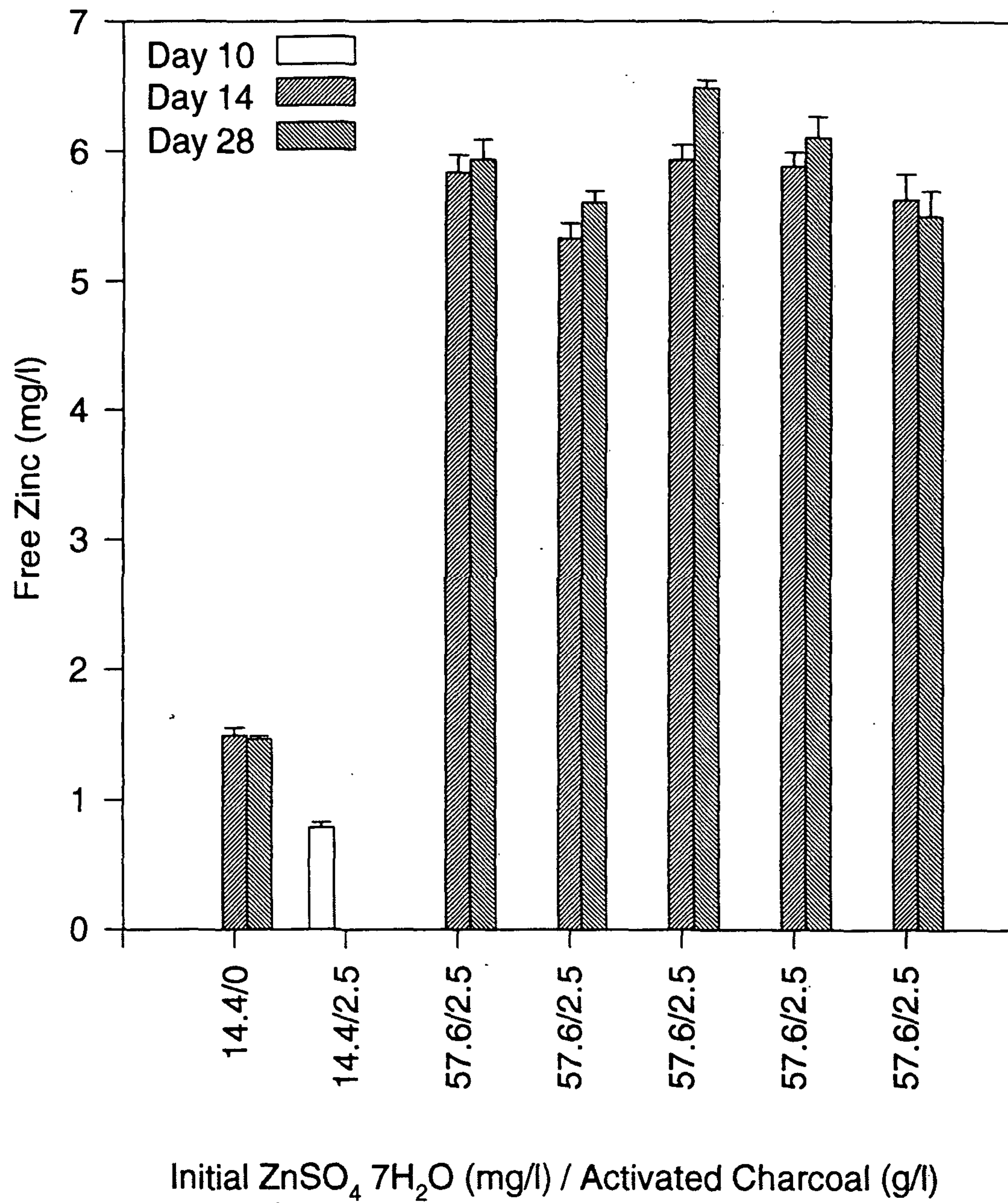


Figure 8. Effect of two $ZnSO_4$ $7H_2O$ and two charcoal concentrations on the amount (mg/l) of free Zn available in initiation media.

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

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.

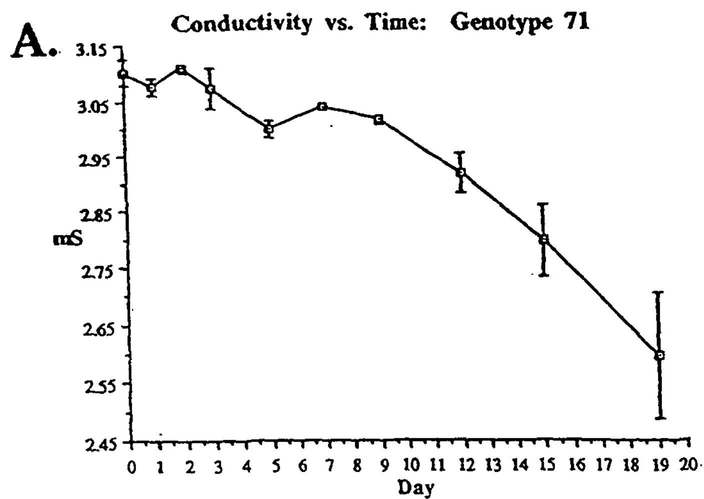
RESULTS AND DISCUSSION

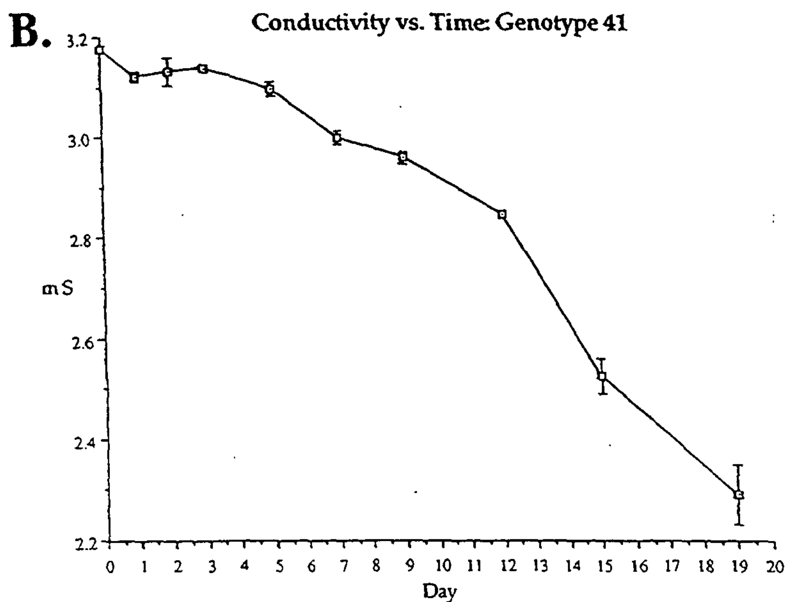
Data for the three genotypes is consistent and within each genotype the standard error bars are fairly small. The electrical conductivity values for the cell suspensions began approximately at 3.15 mS and gradually declined to 2.5 mS indicating that ionic components of the media were being steadily used. 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 NH_4^+ releases H^+ ions. This probably occurs in the first few days until the NH_4^+ uptake is inhibited or becomes unavailable to the cells. As NO_3 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 (NH_4^+ vs. NO_3^-).

	16
NH_4NO_3	603.8
KNO_3	909.9
$Ca(NO_3)_2 \cdot 4H_2O$	236.2
KH_2PO_4	136.1
$MgSO_4 \cdot 7H_2O$	246.5
$Mg(NO_3)_2 \cdot 6H_2O$	256.5
$MgCl_2 \cdot 6H_2O$	101.7
$MnSO_4 \cdot H_2O$	10.5
$ZnSO_4 \cdot 7H_2O$	14.4
$CuSO_4 \cdot 5H_2O$	0.125
$FeSO_4 \cdot 7H_2O$	6.95
Na_2EDTA	9.33
H_3BO_3	15.5
$NaMoO_4 \cdot 2H_2O$	0.125
$CoCl_2 \cdot 6H_2O$	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.





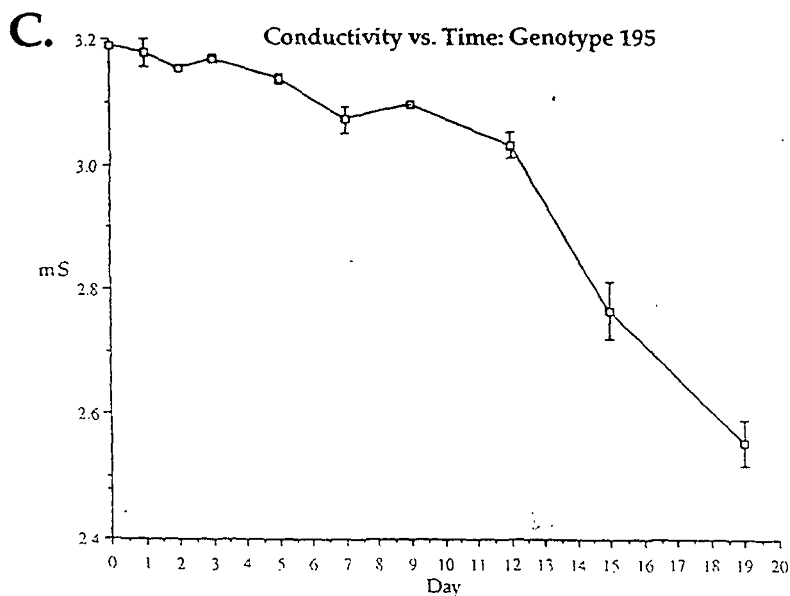
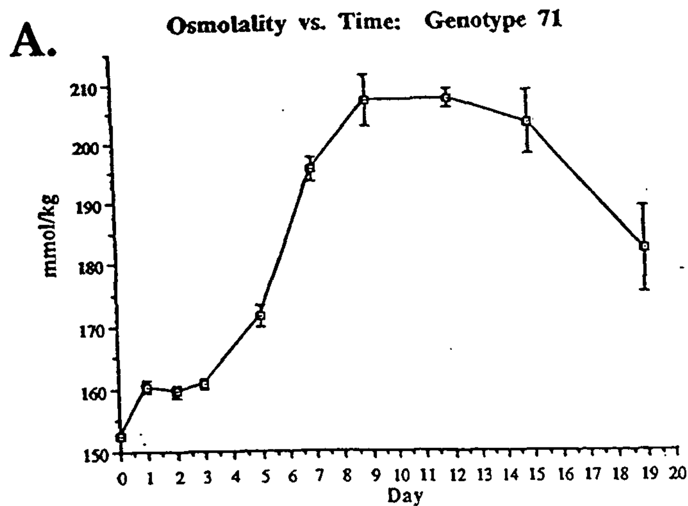
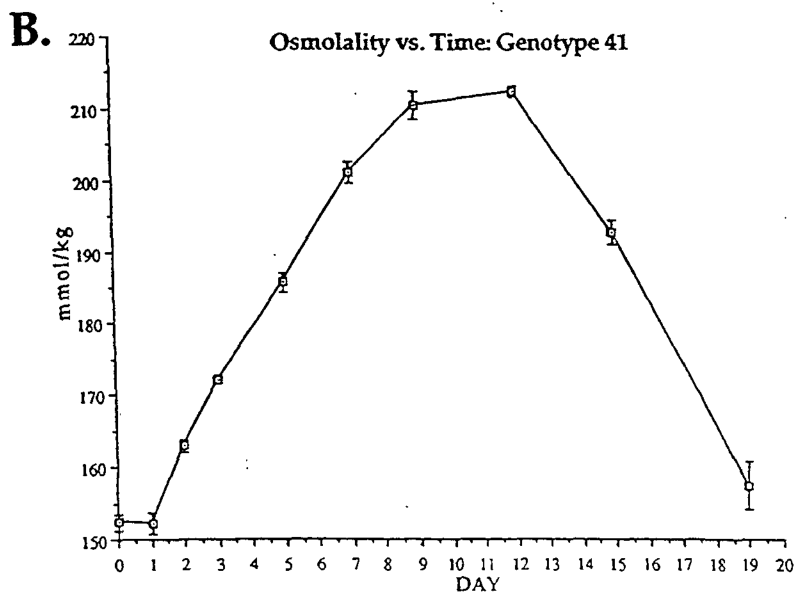


Figure 1. Medium electrical conductivity for three genotypes of Loblolly pine suspension cultures over a 19 day subculture. **A.** Genotype 71 **B.** Genotype 41 **C.** Genotype 195.





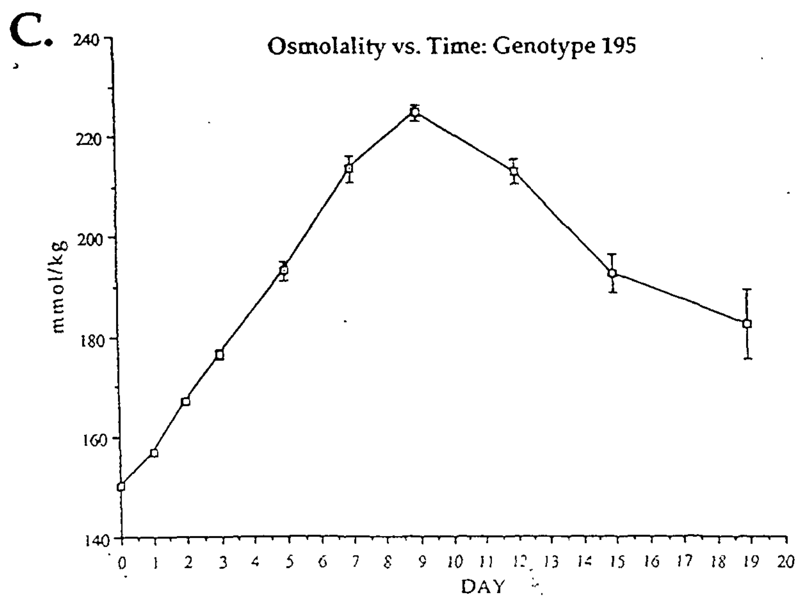
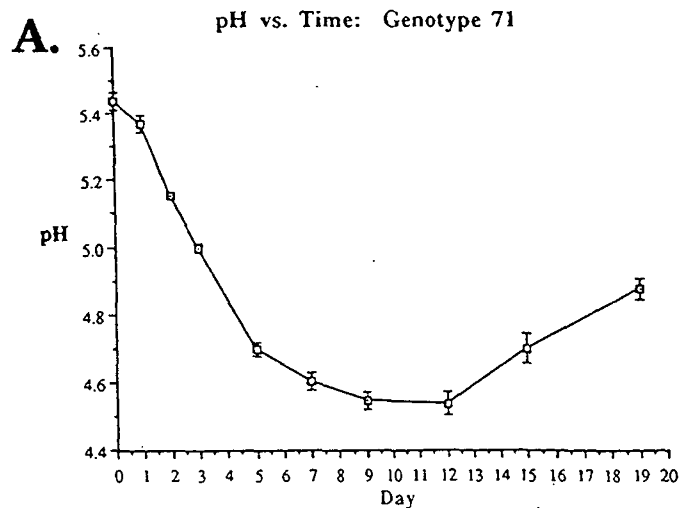
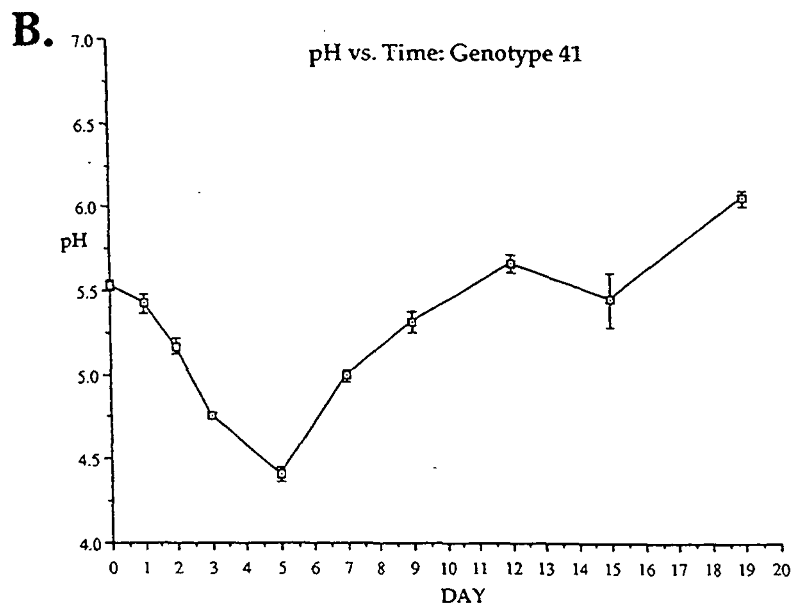


Figure 2. Medium osmolality for three genotypes of Loblolly pine suspension cultures over a 19 day subculture. **A.** Genotype 71 **B.** Genotype 41 C. Genotype 195.





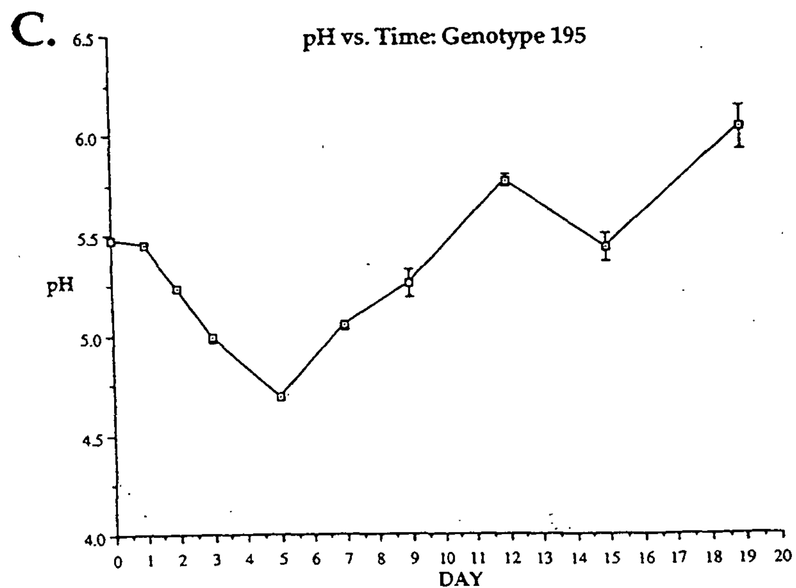
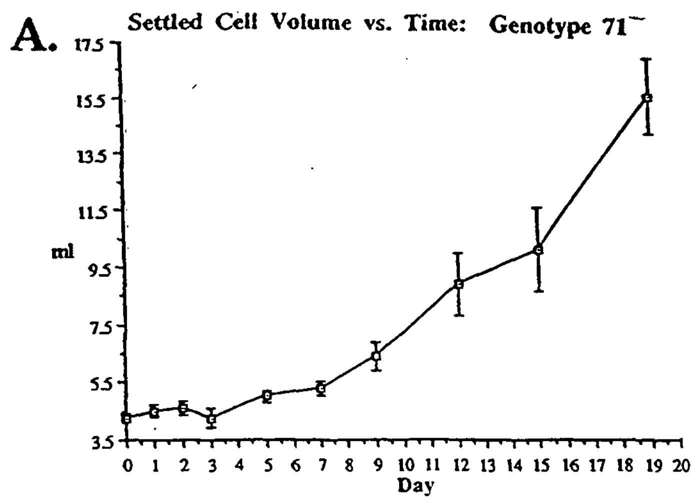
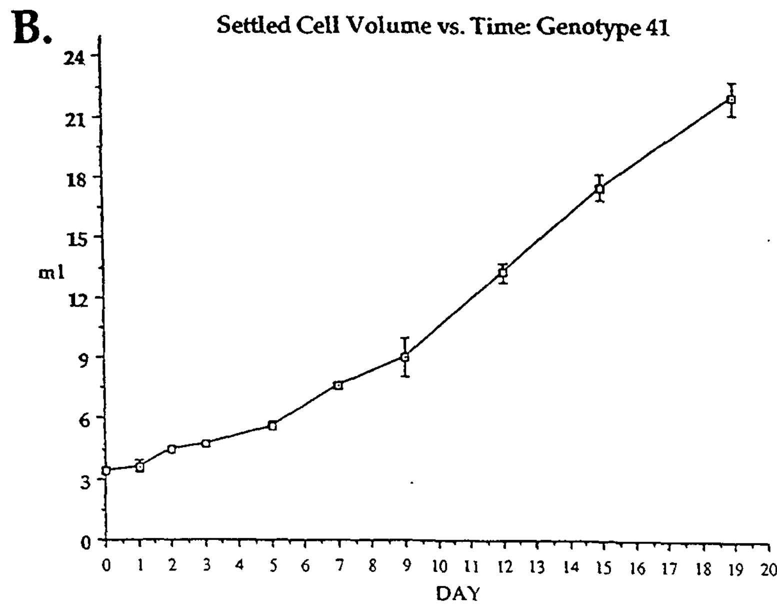


Figure 3. Medium pH for three genotypes of Loblolly pine suspension cultures over a 19 day subculture. **A.** Genotype 71 **B.** Genotype 41 **C.** Genotype 195.





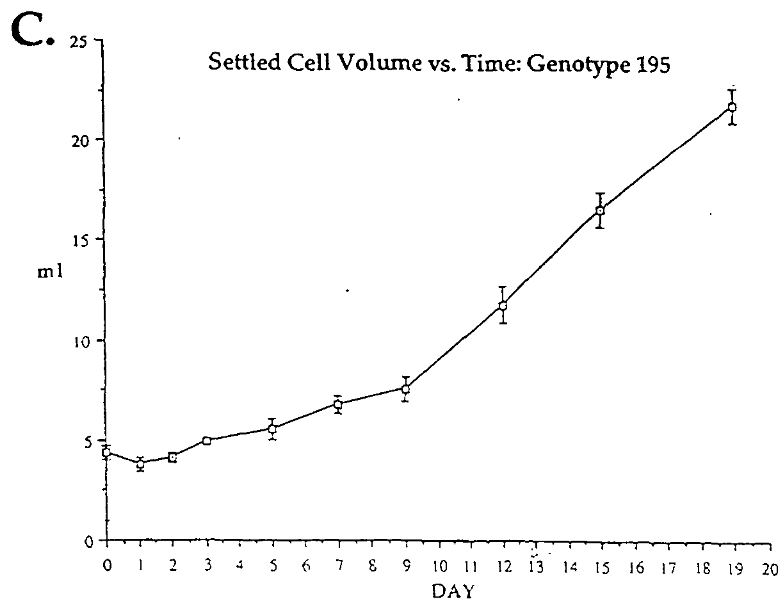
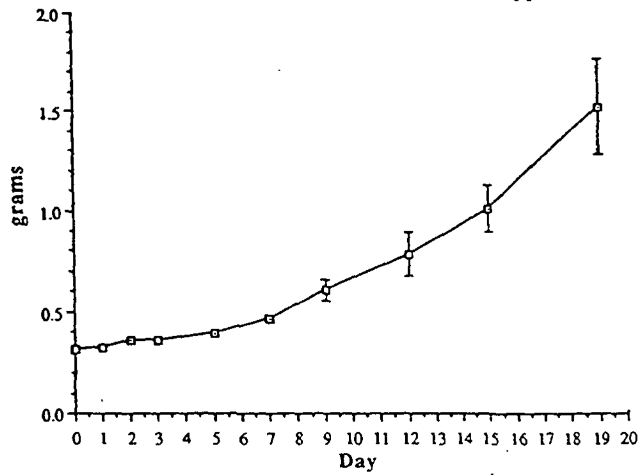
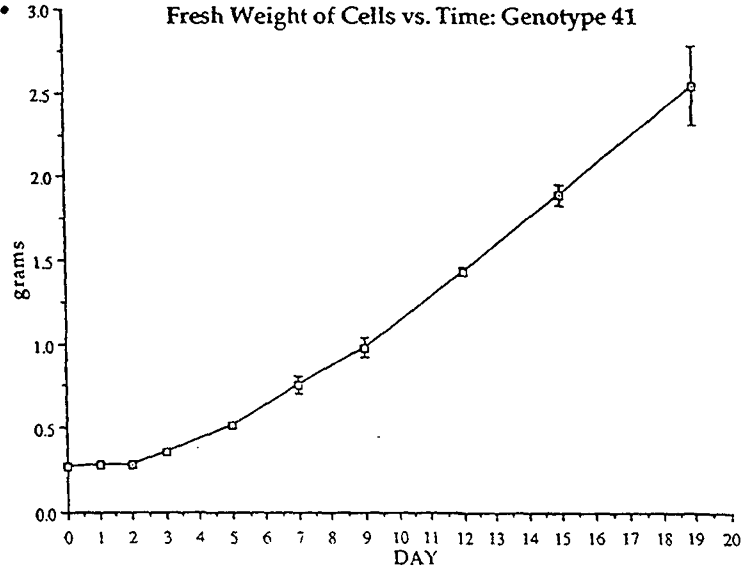


Figure 4. Settled cell volume for three genotypes of Loblolly pine suspension cultures over a 19 day subculture. **A.** Genotype 71 **B.** Genotype 41 C. Genotype 195.

A.



B.



C.

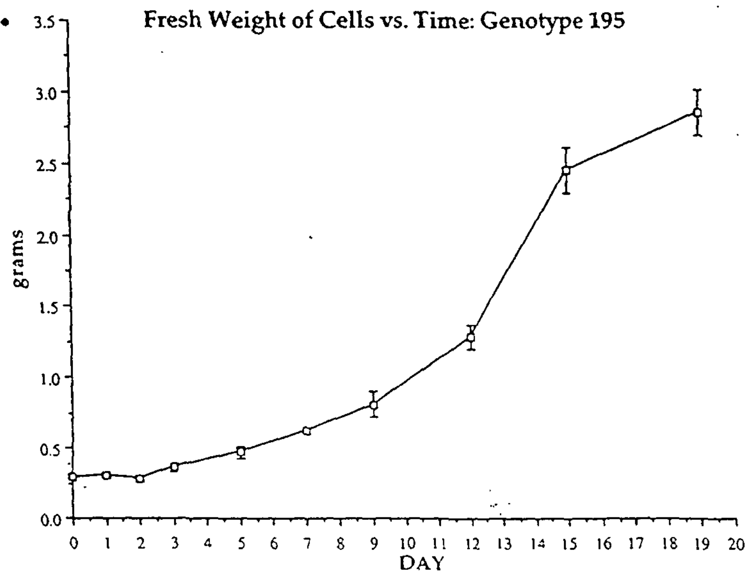
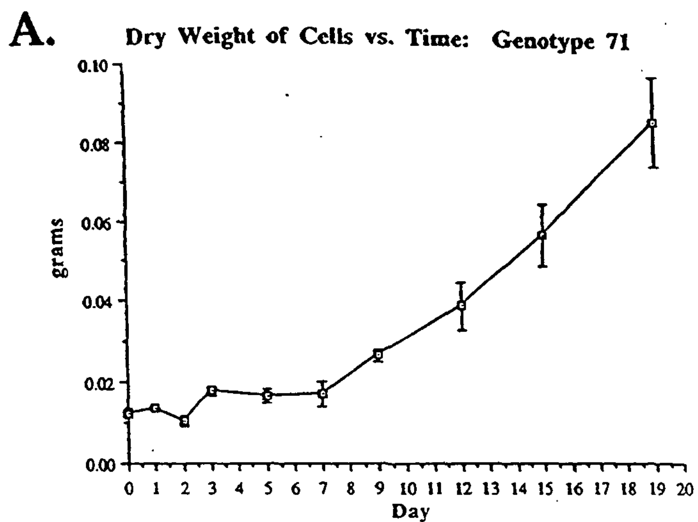
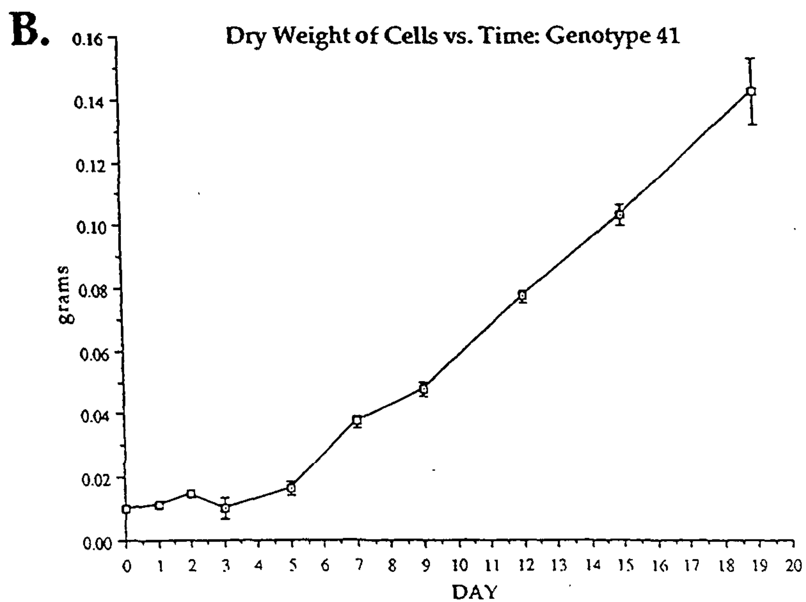


Figure 5. Fresh weight of cells for three genotypes of Loblolly pine suspension cultures over a 19 day subculture. **A.** Genotype 71 **B.** Genotype 41 **C.** Genotype 195.

A.



B.



C.

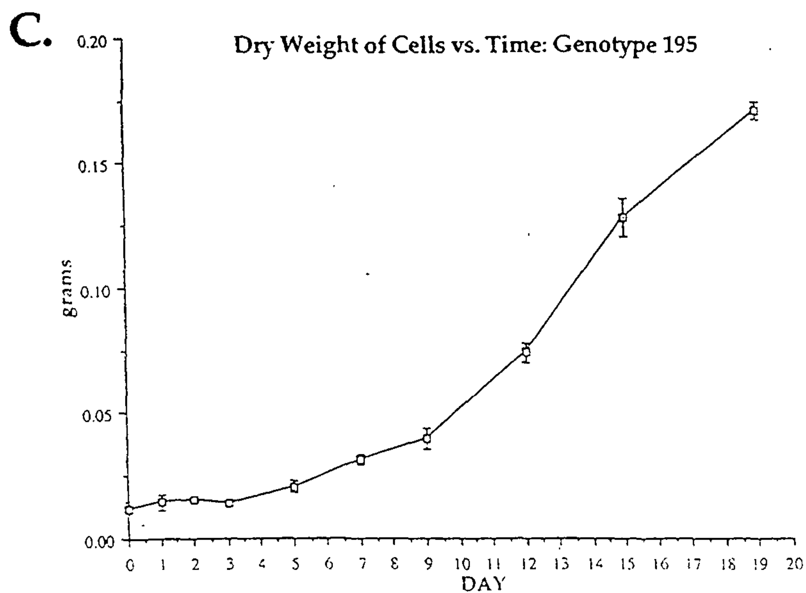
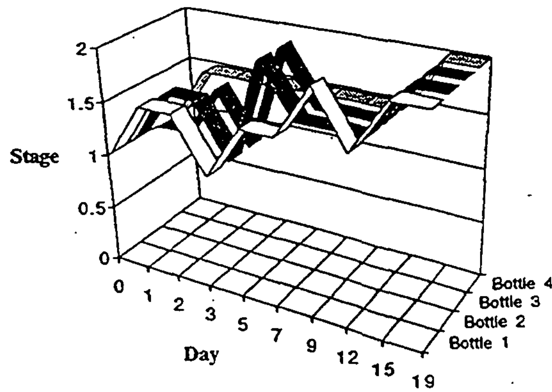
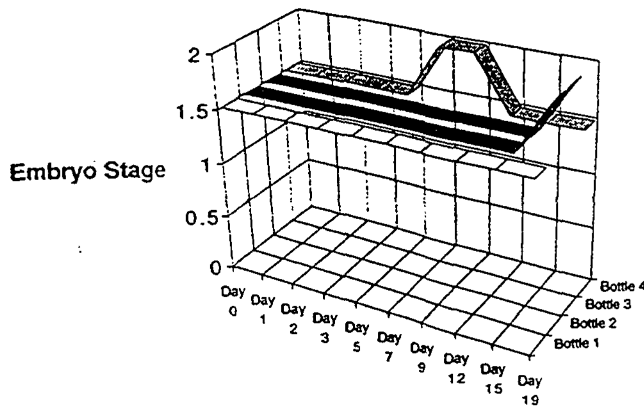


Figure 6. Dry weight of cells for three genotypes of Loblolly pine suspension cultures over a 19 day subculture. **A.** Genotype 71 **B.** Genotype 41 **C.** Genotype 195.

A. Embryo Stage vs. Time: Genotype 71



B. Embryo Stage vs. Time: Genotype 41



C. Embryo Stage vs. Time: Genotype 195

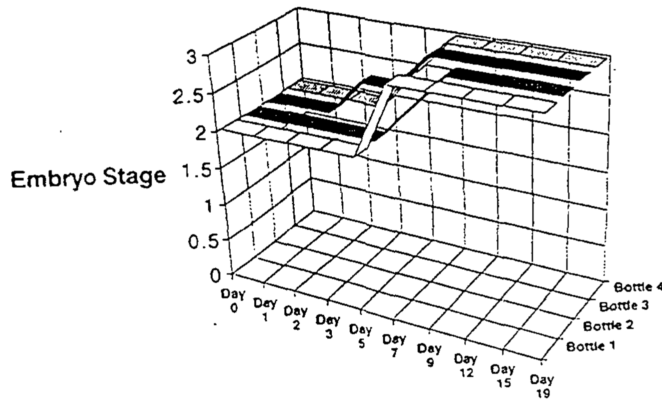


Figure 7. Embryo stage for three genotypes of Loblolly pine suspension cultures over a 19 day subculture. **A.** Genotype 71 **B.** Genotype 41 C. Genotype 195

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - GERMINATION

Gerald Pullman
Barbara Johns
Yolanda Powell
Shannon Johnson

At the time of writing this report approximately 100 loblolly pine somatic embryos have germinated and currently exhibit shoot and root growth in vitro. These somatic seedlings represent the first loblolly pine plants from somatic embryos grown at the institute. Figure 1 shows a typical somatic embryo with shoot and root growth.

Three separate crops of genotype 195 somatic embryos, produced in liquid medium 16 and Development & Maturation media 225 and 240 (Table 1), have shown the ability to germinate into seedlings with shoot and root growth. Two additional genotypes at this time have begun to form germinating embryos with shoot growth but not yet root growth.

Somatic embryos were harvested from Development and maturation medium and placed on a germination medium (55) containing 1/2 MS salts with 2.5 g/l activated charcoal (Table 1). Embryos showed expansion in the hypocotyl region followed by expansion of the cotyledons during the first 4-5 weeks. During the next 4-5 weeks apical primordia appeared and slowly expanded into needle growth. After approximately two months root growth began. Both root and shoot growth continued.

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

All of the somatic embryos 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 producing large numbers of embryos rapidly.

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 - MOLECULAR BIOLOGY

John Cairney
Shujun Chang
Debbie Villalon

We are seeking to reduce lignin synthesis by "antisense" technology. Post-doctoral Senior Technician, Dr. Shujun Chang has cloned an O-methyltransferase cDNA, isolated by us previously from Loblolly Pine, into a plant gene transfer vector. This cDNA has been cloned both in "normal" and "reverse orientation". The reverse orientation or "antisense" construct will be transferred into Sweetgum, Cottonwood and Tobacco cells. Transgenic plants will be regenerated and lignin quantity and quality will be assayed.

The production of stronger, healthier trees would be enhanced by and understanding of the biochemical pathways involved in transmitting information on environmental changes to genes involved in defense and repair. We had isolated previously clones of genes we had defined as drought responsive. In recent experiments we have shown that certain of these genes are also switched on by hormones such as ABA and Jasmonic Acid. Levels of these hormones rise in plants subjected to environmental stresses.

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, a plant defense protein, and to examine the regulation of the gene which encodes it. We have generated DNA fragments representing the control regions of this gene using Polymerase Chain Reaction (PCR) and are cloning these into plant vectors for transfer into plant cells. In addition we are cloning the "coding regions" of our Proteinase Inhibitor gene into "expression vectors" with a view to analyzing how effective it is against a variety of proteinases.

We will conduct this and a number of future studies in the model plant *Arabidopsis thaliana*. This plant grows rapidly and is easy to manipulate genetically. We have succeeded in regenerating *Arabidopsis* plants from tissue culture, a necessary capability for gene transfer studies. In preliminary gene transfer studies we have demonstrated that a reporter "GUS" gene transferred into *Arabidopsis* cells by *Agrobacterium tumefaciens* was taken up and expressed transiently. Currently more extensive gene transfer and plant regeneration studies are being conducted.

To enhance our understanding of the molecular events responsible for somatic embryo development we are initiating a project to assay gene expression at different stages in the developmental program. We wish to compare somatic and zygotic processes. We have isolated zygotic embryos from all eight stages and will be collecting a complementary set of somatic

embryos. Our intention is to use the technique of Differential Display to reveal differences in gene expression. The immediate goal of this progràm is to refine the technology so that it can be used with such small amounts of starting material.

HARDWOODS

TECHNICAL PROGRAM REVIEW
FY 94-95

Project Title:	MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS
Project Code:	HRDWD
Project Number:	3223-03
Division:	Chemical and Biological Sciences
Project Staff:	R.J. Dinus and C.J. Stephens
FY 94-95:	$132,500

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 herbicide tolerance and other valuable traits into commercially important hardwoods, and for effective mass propagation, testing, and release of genetically modified trees.

Maintenance of cottonwood clones in the greenhouse continues as per past reports. Additional ramets of model clones C175 and K417 as well as elite clones St75, St66, and WV96 will be regenerated from culture and used to replace older ramets as needed. Preliminary arrangements have been made to acquire cuttings of three hybrids derived from crosses between Populus deltoides and P. trichocarpa. All are triploids and sexually sterile. Permits for testing transgenic plants derived from such clones can be secured with far less difficulty than would otherwise be the case. Maintenance, replenishment, and accession are necessary for continued availability of explants for ongoing and future research.

Research on establishment and multiplication of cultures from internode explants (greenhouse) remained at a low level. A lethal dose assay completed since the last report indicates that modest doses of kanamycin, an antibiotic selective for transformed materials clearly limits regeneration from K417 internodes. Transformation with internodes could therefore provide a means for transforming clones not easily manipulated via the Leaf Section System. In addition, a modest experiment was initiated to provide material for replacing older greenhouse ramets of C175, K417, St75, St66, and WV96.

Efforts to extend the Leaf Section System to additional clones continued at the same modest level of activity as last year. One experiment testing applicability to K417 was established;

the standard system is being compared to two alternative protocols from the literature. To date, the standard system is outperforming the others. This and a repeat trial must be completed before firm conclusions are drawn.

Tissue cultures transformed with the Monsanto Corp. gene for enhanced auxin synthesis and constitutive promoter continue on a maintenance/multiplication regime, with the intent of ensuring a continuing supply of material for later reconfirmation of transformation.

Similarly, work on reconfirming transformation of the aforementioned cultures via Southern blotting was also stopped. Since regeneration of transgenic plants was not likely, resources were reallocated to new experiments on transformation with the same gene coupled to a controlled heat shock promoter and with a GUS gene coupled to the same constitutive promoter.

Student research on transformation with the auxin synthesis gene and controllable promoter continued through June, 1994, when the individual graduated. The final experiments mentioned in the last report did not yield transgenic plants. Several shoots were produced, but none survived on selective media. All experiments were plagued with excessive and debilitating contamination from residual Agrobacterium tumefaciens (At).

Since then, four transformation experiments have been established; two with the auxin synthesis gene and controllable promoter and two with the GUS gene and a constitutive promoter. Preliminary findings are available from the first two experiments; one each with the auxin synthesis and GUS genes. Control leaf sections not exposed to At or the selective antibiotic, kanamycin, are producing callus, primordia, and shoots in accordance with expectations. Standards not exposed to At but cultured on media containing kanamycin, also as expected, are dead or near death. Sections exposed to At but not kanamycin formed callus and primordia later than controls, and are now yielding shoots for testing on selective medium. Roughly 75 percent of sections exposed to At and kanamycin have small but significant patches of living, potentially transformed tissue. Half of these sections are being maintained on selective medium and half on nonselective medium. The latter approach is being used to heighten probability of regenerating transgenic plants. Any shoots formed under this nonselective circumstances will be challenged with kanamycin later. Performance of all leaf sections and harvested shoots is being monitored across time, with provision for capturing calli and/or leaves as sources of DNA for Southern blotting.

As noted above, student research on transformation in 1993-94 was plagued by severe residual At contamination. In the current transformation trials, weekly observations are being used to quantify contamination across time, with action taken to prevent or remedy outbreaks. To date, residual At contamination has been far lower than that encountered by the student, an outcome no doubt due to the skills and commitment of our technical staff. Modest levels of contamination, however, have been observed. As a result, we began testing cefotaxime, a potential substitute for carbenicillin, the antibiotic normally used to eliminate residual At. A lethal dose assay now in progress suggests that cefotaxime will clear cultures of At at doses described in the literature (100 - 250 mg/l), with little or no effect on cottonwood leaf sections. To date, leaf sections exposed to cefotaxime concentrations ranging from 25 to 500 mg/l are

producing callus and primordia at frequencies comparable to those of controls. Cefotaxime offers some advantage over carbenicillin in being less subject to degradation by light and enzymatic action, critical factors given the lengthy culture periods required for cottonwood transformation and regeneration. A decision to use cefotaxime routinely in lieu of or in tandem with carbenicillin will not be made until harvestable shoot numbers have been evaluated.

Three experiments were initiated to extend transformation to additional explants and clones. K417 internodes taken from the greenhouse perform well in culture, whereas its leaf sections are less responsive than other clones; e.g., C175. As a result, a trial testing responsiveness of internodes collected from K417 plants grown in sterile culture was undertaken. Results to date indicate that internodes from culture are more responsive than those from the greenhouse. Callus, primordia, and shoots form earlier, and yields of harvestable shoots are on a par or greater than those for internodes from the greenhouse. After 49 days in culture, roughly half the lifetime of a regeneration experiment, 100 percent of the explants are free of contamination, 100 percent have formed callus and primordia, and 91 percent have produced harvestable shoots. Internodes from culture may thus provide a suitable vehicle for transformation, one advantage being that they are available in large numbers throughout the year. A workable procedure for using them will also minimize losses to bacterial contamination, that frequently occur with greenhouse internodes, particularly in late summer and fall. Two additional trials have been established to test reproducibility of the procedure with K417 and to evaluate applicability to another clone.

PLANNED ACTIVITY THROUGH FISCAL YEAR 1994-95:

* Effect transformation of model clone C175

* Insert a benign marker gene (GUS) to proof and refine the transformation protocol. Repeat until an efficient protocol for transformation is obtained.

* Insert an auxin synthesis gene coupled to a controllable promoter for purposes of studying auxin effects on growth and fiber formation. Repeat until an efficient protocol for transformation is secured.

* Regenerate transgenic plants from transformed cultures. Repeat until regeneration of transformed plants has been confirmed.

* Confirm transformation via biochemical and molecular assays; e.g., Southern blotting.

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

FUTURE RESEARCH ACTIVITY:

* Extend transformation to other clones and/or species.

* Expand system to include transformation with genes of more immediate commercial value.

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

Following is a list of 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

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:

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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
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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 Matters
Title: President-IPST

Gary 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:**

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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

TABLE OF CONTENTS

COVER PAGE i

TABLE OF CONTENTS ii

ABSTRACT iii

PROJECT DESCRIPTION 1
- INTRODUCTION 1
- OBJECTIVES 8
- HYPOTHESES 9
- RATIONALE AND SIGNIFICANCE 9
- RESEARCH PROCEDURES 10
  - Experimental Approach 10
  - General Research Methods 15
- TENTATIVE SCHEDULE 17

FACILITIES AND EQUIPMENT 17
COLLABORATIVE ARRANGEMENTS 18
REFERENCES 18
VITAE AND PUBLICATION LIST(S) 25
BUDGET 33
BUDGET JUSTIFICATION 35
CONSULTING CONFIRMATION LETTER 36
REVIEW OF PREVIOUS SUBMISSION 37
REQUIRED FORMS 41

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

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Shaded Area for EPA Use Only

9. Date Received

10. Proposal Number

11. Committee Assignment

12. Comments

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

Ard, T. A., R. J. Dinus, S. G. Donkin, and D. B. Dusenbery. 1994. The use of a nematode *Caenorhabditis elegans*, for biomonitoring. In: Procs. 1994 TAPPI International Environmental Conference, Book 2, pp 885-892. April, 1994, Portland Or.

Becwar, M. R. and G. S. Pullman. 1994. Somatic embryogenesis in loblolly pine (*Pinus taeda* L.). In: Somatic embryogenesis in woody plants (Eds. S. Mohan Jain, P. K. Gupta, and R. J. Newton), Kluwer, The Netherlands. **(in press)**

Cairney J. , Chang S. , Dias D., Funkhouser E. A. , Newton R. J. 1993. cDNA Cloning and Analysis of Loblolly Pine Genes Induced by Drought Stress. Proceedings, 22nd Southern Forest Tree Improvement Conference 14-17 June, Atlanta, Georgia. **(in press)**

Cairney J., Newton R. J., Funkhouser E. A., Chang S. 1994. Nucleotide Sequence of a cDNA from *Atriplex canescens* (Pursh.) Nutt.: a homolog of a Jasmonate-Induced Protein from Barley **(in preparation)**

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Walker, C. C., T. J. McDonough, R. J. Dinus, and K. -E. L. Eriksson. 1994. Catalytic reactions in a polymeric model system for hydrogen peroxide delignification of pulp. In: Procs. Am. Inst. of Chemical Engineers, Spring Meeting, April 17-21, 1994, Atlanta GA. (**in press**).

Walker, C. C., T. J. McDonough, R. J. Dinus, and K. -E. L. Eriksson. 1994. An evaluation of three iron-based biomimetic compounds for their selectivity in a polymeric model system for pulp. To be presented at TAPPI / USDA Biological Sciences Symposium. October 2-6, 1994, Minneapolis, Mn.

Walker, C. C., T. J. McDonough, R. J. Dinus, K.-E. L. Eriksson. 1994. **Catalytic reactions in a** polymeric model system for hydrogen peroxide delignification **of pulp. Presented at** AIChE Spring Meeting, April 17-21, 1994. Atlanta, GA

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.

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.

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.

Lois Ford (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 this quarter have centered on the pulping, handsheet production and testing of wood chips treated with Cartapip for three and five week periods. Similar work was done on chips incubated for three and five week time periods, as well as time zero control chips. Figure 1 shows the completion of extractive work from the spring quarter. The three week samples show the greatest difference in extractive levels between Cartapip treated and incubated non-treated controls. The greatest differences in strength and pulp properties are generally seen after five weeks in contrast to the extractive levels. The properties most influenced by Cartapip treatment and incubation time are: fines content in the pulp, tear strength of handsheets, and handsheet density. The fines content of the Cartapip treated samples decreased more than control samples incubated for the same amounts of time. The density of the sheets produced from Cartapip treated chips also decreased. This would be expected with a decrease in fines content. Perhaps the most dramatic change can be seen in the tear strength, where Cartapip treatment increases the strength by 1.15 mNm^2/g after three weeks and by a factor of 1.3 after five week treatment when compared to controls incubated for the same amount of time. (See figures at the end of this section.)

Rene Kapik (Ph. D.) *
Title: A study of plant growth regulators during zygotic embryogenesis in loblolly pine.
Advisor: Ron Dinus

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: In preparation
Advisor: Jerry Pullman
Summary: Currently I am beginning my Ph.D. research proposal (A490) regarding the use of activated charcoal in tissue culture medium. The tentative focus will be on three hypotheses related to the observation that "acid-washed" activated charcoal supplied through Sigma performs well whereas non-acid-washed charcoal (also Sigma brand) does not. The three hypotheses relate to sorption of organics and the sorption of, or possible release of, metal ions. In the course of addressing these hypotheses more will be learned about the surface character and sorption behavior of activated charcoal.

Colleen Walker (Ph. D.)
Title: Selectivity of iron-based catalysts in a polymeric model system for biomimetic bleaching. (Colleen is in the process of completing the last steps of submitting her thesis for publication by the institute.)
Advisor: Ron Dinus
Summary: Discovery and subsequent 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 three systems investigated were ferrous sulfate, ferrous ion chelated with ethylenediaminetetraacetic acid (EDTA), and hemoglobin, all in the presence of hydrogen peroxide. A unique polymeric, homogeneous model system has been used to represent wood pulp. Lignosulfonate 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 for each biomimetic catalyst. The two were then compared to determine the selectivity of each catalytic system. Rates of chain scission were also compared. Hemoglobin was found to be the most selective for lignosulfonate degradation over HEC degradation.

The amount of hydrogen peroxide decomposed to oxygen vs. that consumed in reactions was monitored for each of these systems. Hemoglobin was found to be the most effective for oxidizing lignosulfonate. Fe-EDTA resulted in the greatest loss of hydrogen peroxide as oxygen.

During these reactions the production of hydroxyl radicals was measured using a chemiluminescence assay, which was a slightly modified version of a published procedure. Although hemoglobin interfered with this assay, results from the ferrous sulfate and chelated ferrous catalysts were obtained. Rates of hydroxyl radical production in the ferrous system were directly related to bond cleavage of lignosulfonate. This was not the case for Fe-EDTA.

When lignosulfonate and HEC were simultaneous exposed to hydrogen peroxide and the same catalysts, the formation of a high molecular weight product was observed. This product is the result of a condensation reaction between the lignin and cellulose models. Its formation is significant inasmuch as it models a counterproductive process that may be responsible for the limited effectiveness of enzymatic delignification systems using lignin peroxidases.

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: The objectives of this thesis are to demonstrate that the RAPD PCR technique can quantify genetic structure in populations of *Pinus elliottii* and use this ability to investigate and monitor changes in populations of a tree improvement program.

Figure 1. Relationship Between Extractive Content and Cartapip Residence Time

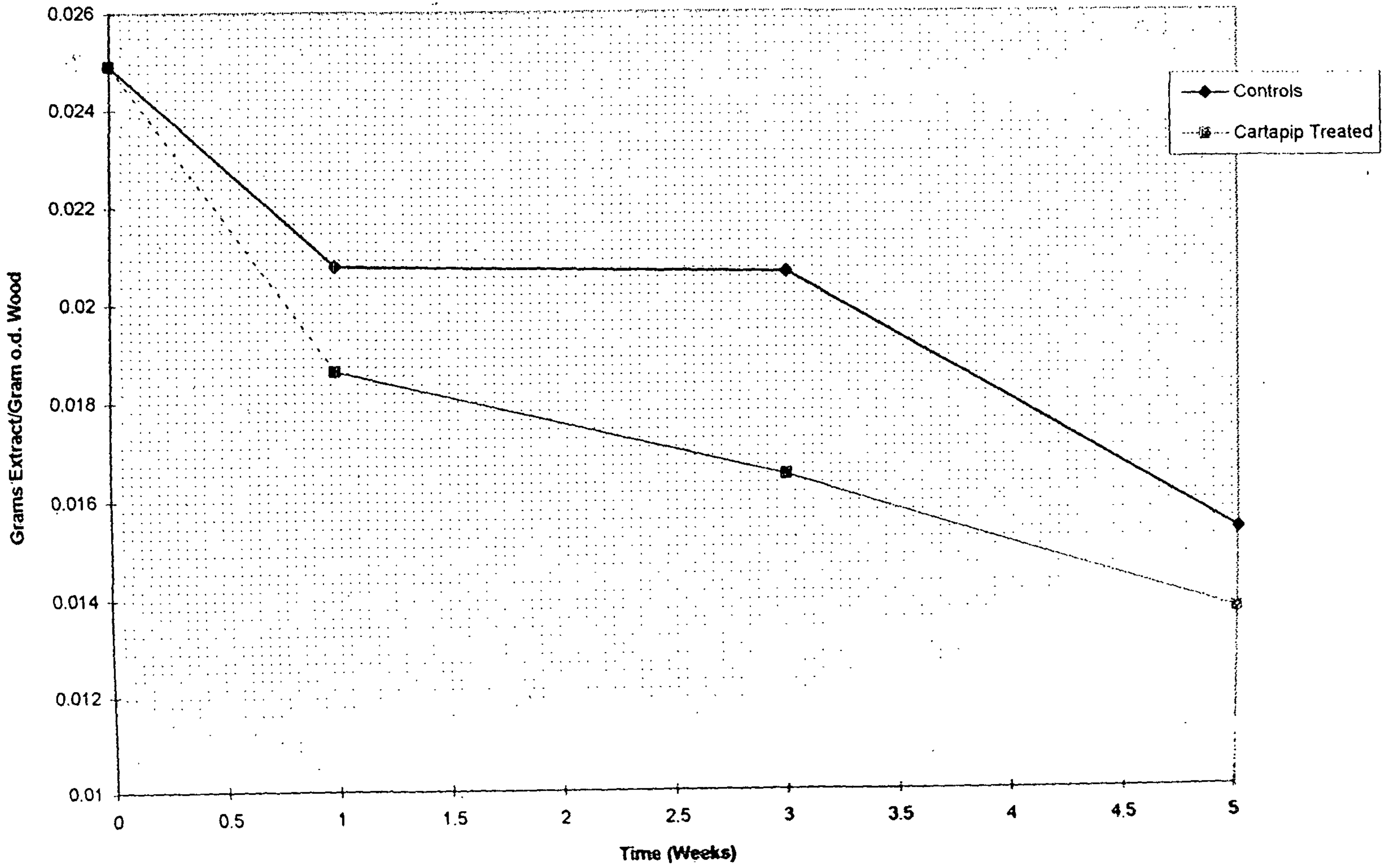
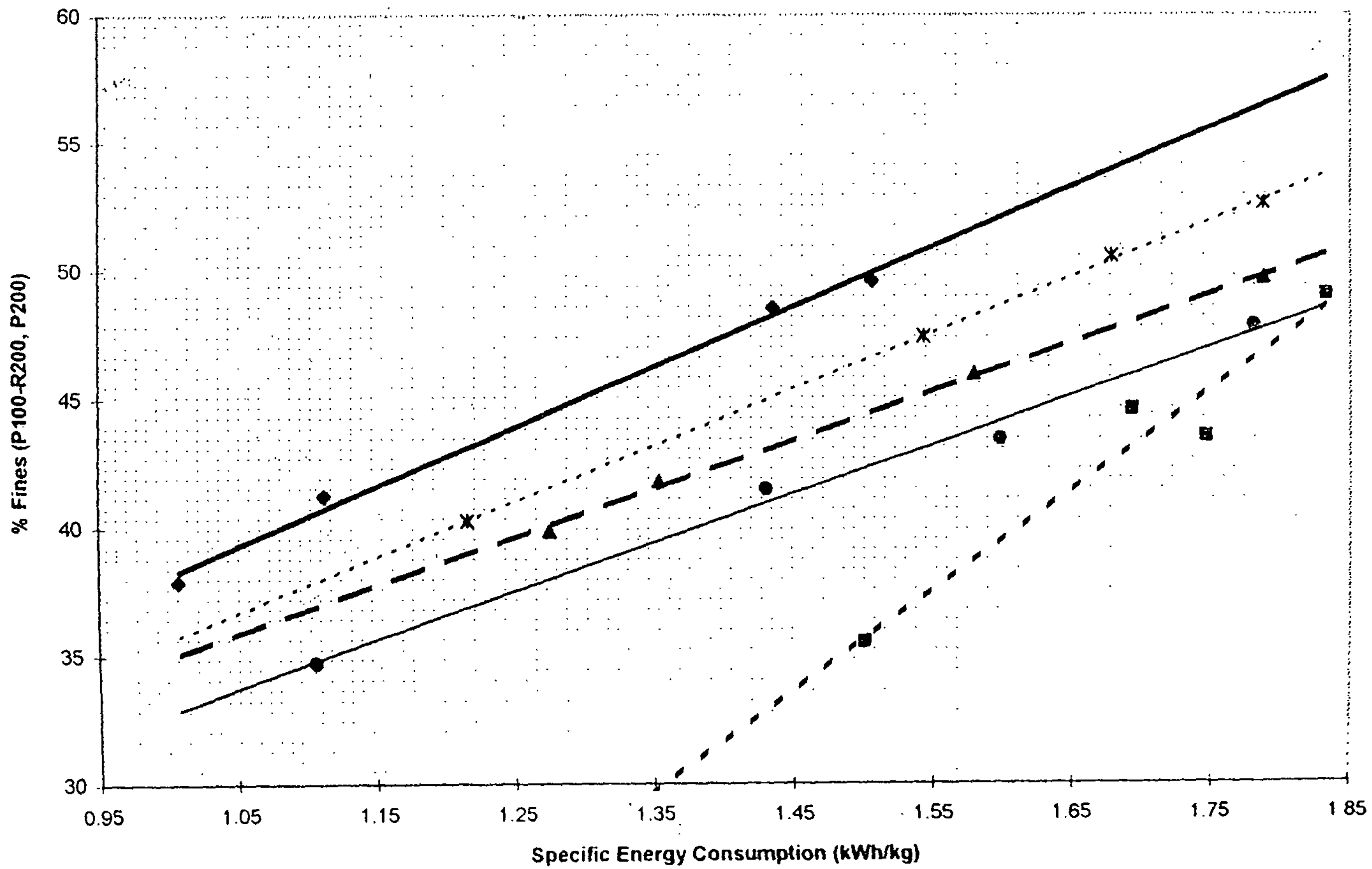
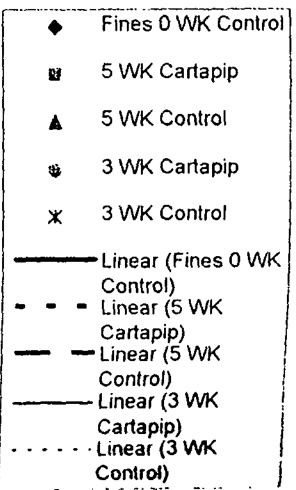
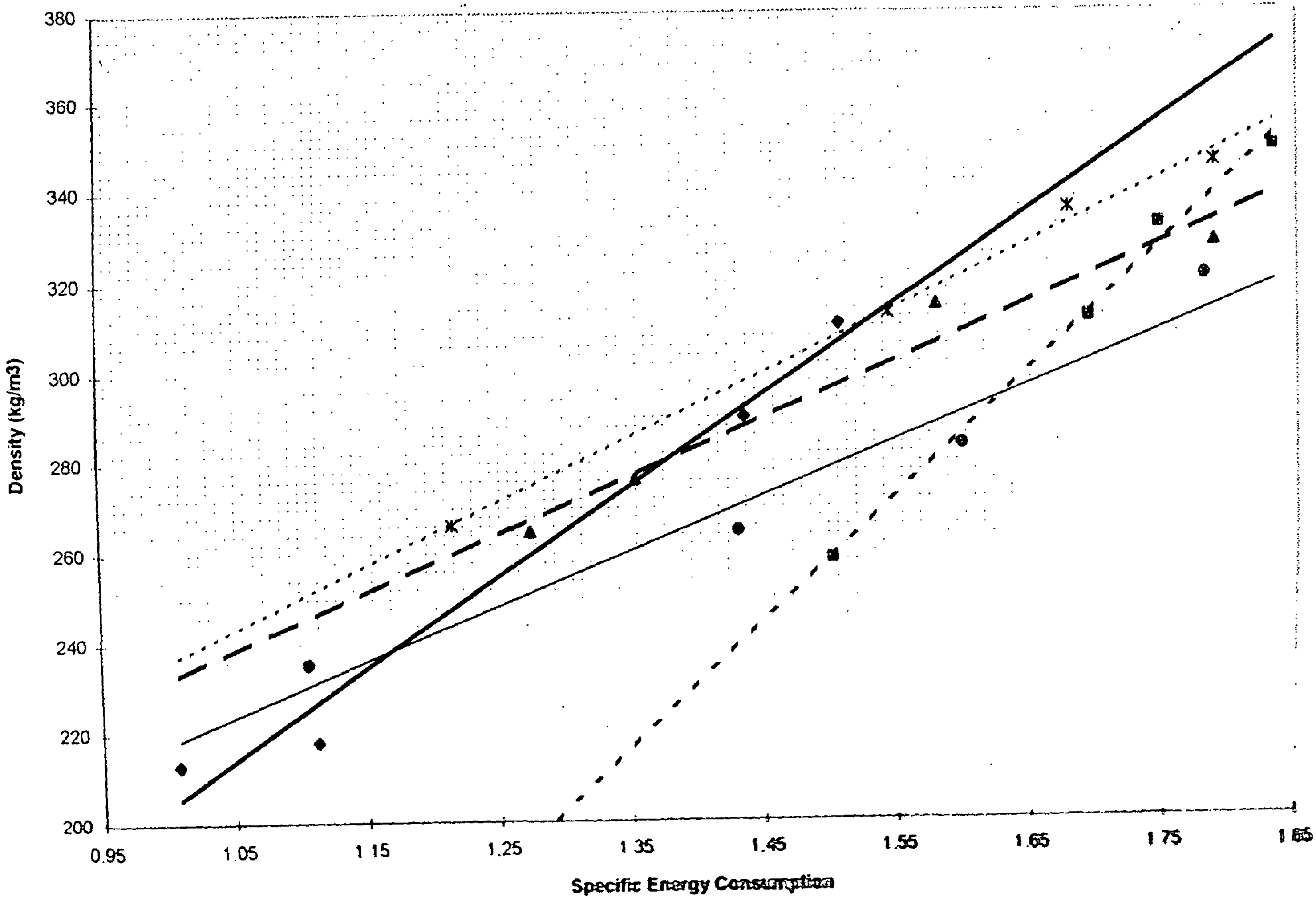


Figure 3. Fines Generation with Energy Consumption







Density Energy Relationship
Density (kg/m3)
380
360
340
320
300
280
260
240
220
200
0.95
1.05
1.15
1.25
1.35
1.45
1.55
1.65
1.75
1.85
Specific Energy Consumption
D 0 Wk Control
D 5 Wk Cartapip
D 5 WK Control
D 3 WK Cartapip
D 3 WK Control
Linear (D 0 Wk Control)
Linear (D 5 Wk Cartapip)
Linear (D 5 WK Control)
Linear (D 3 WK Cartapip)
Linear (D 3 WK Control)

Figure 6. Tear Specific Energy Consumption Relationship