FOREST BIOLOGY

ANNUAL PROGRAM REVIEW

MARCH 19 - 20, 1996

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ANNUAL PROGRAM REVIEW

March 19 - 20, 1996

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FOREST BIOLOGY ANNUAL RESEARCH REVIEW
AGENDA

Tuesday, March 19, 1996
Forest Biology Annual Program Review (Room 114)

8:00 A.M.	Coffee and Donuts	
8:30	Welcome, introduction, antitrust statement	Lazar

8:40	Hardwoods - Cottonwood Transformation
	    Background & Supporting Research
	    Transformation Experiments
	    Confirmation of Transformation
		Cairney

10:00	Break	

10:15	Molecular Biology - Softwoods & Hardwoods
	    Differential Display
	        Somatic Embryo Improvement
	        Gene Expression Studies
	    Sterility
		Cairney

12:00	Lunch	

Forest Biology PAC Meeting (Room 173)

1:00	Softwood Embryogenesis
	    Initiation
	    New Culture Performance
	    Cryogenic Storage
	    Conversion
		Pullman

3:00	Break	
3:15	Grant Proposal Activity, Student Research, Funding for Hardwood Program	Pullman, Cairney

	New Programs and Personnel:
	    Faculty, Differential Display, Lignin,
	    Sterility, Issues
		Pullman,
		Cairney

5:30	Dinner (Research on Bleaching)	Ragauskas

Wednesday, March 20, 1996

Forest Biology PAC Meeting Continued (Room 171)

8:00 A.M.	Coffee and Donuts	
8:30	New Organizational Structure at IPST New Faculty	Malcolm

8:45	RAC Developments,
	    Patent / Licensing
	    Sub-Committees	

9:15	Forest Biology PAC Presentation to RAC	Lazar
9:45	RAC / PAC Interactions	Lazar
10:00	Break	

10:15	Forest Research Initiatives
	    AF & PA
	    PAC Sub-Committee
		Malcolm, Canavera
		Lazar

10:35	TAPPI Biological Sciences Symposium	Lazar
11:00	Adjourn (Lunch will be available at 12:00)	

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SOFTWOODS

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS
TECHNICAL PROGRAM REVIEW
February, 1995 - February 1996

Project Title: MASS CLONAL PROPAGATION OF IMPROVED CONIFERS
Project Code: SFTWD
Project Number: F-010
Division: Chemical and Biological Sciences
PAC: Forest Biology
Project Staff: Gerald Pullman, John Cairney, Luis Destefano, Barbara Johns, Shannon Johnson, Yolanda Powell, open
FY 95-96 Budget: $455,000
Long Range Research Need: Develop an assured supply of competitive virgin fiber.
        Increase growth to maximize yield per acre per year.
        Improve surviability of planted species.
        Improve fiber characteristics to match product and process requirements.

PROGRAM OBJECTIVE:

Develop methods suitable for commercial production of high quality somatic loblolly pine seedlings.

SUMMARY OF RESULTS:

Forest Biology Faculty brought in approximately $ 181,000 in outside funding during the past year.

Embryogenic Initiation rates from immature seeds of high value loblolly pine showed a major improvement from 1% to 16% across many mother trees.

A190 student research developed adsorption model for adsorption of 2,4-D (plant hormone) by activated carbon in tissue culture medium. Model helped to improve initiation rates.

The Institute's first crop of loblolly pine somatic seedlings has been produced. Germinated loblolly pine somatic embryos underwent successful conversion to growth in an open greenhouse.   We currently have 61 converted plants and an additional 56 undergoing conversion from genotype 195. An additional 29 plants are undergoing conversion from a second genotype (230).

A cryogenic storage system for storage of loblolly somatic embryo cultures in liquid nitrogen has been purchased and is operating.

Differential display technique is working well in the laboratory. Currently in use for comparison of somatic and zygotic embryos revealing for the first time details of differential gene expression in developing conifer embryos.

Medium osmolarity and hormone content was shown to regulate gene expression of early-stage embryos in culture.

Novel gene regulatory sequence identified in a stress related gene, function under investigation.

"Sense" LP1 (OMT-homolog) transformed into tobacco. Putative transformants under analysis.

Differential display used to identify mRNA species specific to stem, needle, and roots of unstressed loblolly pine. These have been isolated as cDNA's and will be sequenced and analyzed. This is a route towards useful promoters for gene expression.

Initial experiments show floral specific genes in loblolly pine may be cloned by PCR.

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - INITIATION

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - INITIATION

Gerald Pullman
Shannon Johnson
Christine Estes
Barbara Johns
Yolanda Powell
Theresa Massey

Summary

We are happy to report that loblolly pine initiation rates have been improved by at least an order of magnitude. Through a combination of 1/2 P6 Salts, activated carbon at 50-100 mg/l, extra added copper and zinc, 1.5% maltose, 2% myo-inositol, 500 mg/l case amino acids, 450 mg/l glutamine, Gelrite gelling agent at 1.5-2 g/l, and hormones consisting of 2 ppm NAA, 0.45 ppm BAP and 0.43 ppm Kinetin, initiation rates have ranged from 3-33% across many genotypes. This current medium has resulted from numerous experiments over the past 2.5 years. Experiments identified at least four blocks that needed to be corrected to improve initiation rates. Those factors are as follows:

1) Addition of activated charcoal (AC) to stimulate extrusion.
2) Activated carbon inhibits initiation at levels above 100 mg/l . Use low levels (50-100 mg/l) of AC to allow both increased extrusion and initiation to occur.
3) High hormone levels (220 ppm 2,4-D) added to compensate for adsorption by AC were too high and provided free hormone levels probably inhibitory to somatic embryo growth.
4) Our earlier Gelrite levels of 4 g/l (based on observations of extrusion responses) were inhibitory to culture initiation. Gelrite levels between 1.5-2 g/l appear to be optimal.

We now obtain initiation levels high enough to conduct statistical evaluations of experimental treatments. Previously statistical evaluations were only possible for effects of treatments on extrusion % or growth of somatic embryos used as explants.

During the 1995 Summer 436 cultures were initiated from approximately 6400 immature seeds providing an overall initiation rate from poor and good treatments of 6.8%. When the best two media are considered (media 539 with 1.6 gelrite or medium 505 with 2.0 grams of gelrite), initiation across 16 mother trees was 16%.

Introduction & Results

The initiation of an embryogenic culture or embryo suspensor mass (ESM) is the first step in cloning the embryo(s) from a valuable conifer seed. The process in loblolly pine starts with an immature seed. The seed is sterilized and the seed coat removed to expose the ovule or female gametophyte which contains the early-staged embryo(s). The whole female gametophyte (megagametophyte) is placed on a chemically defined medium and incubated in the dark at 22-24 C. The process of initiation then occurs in several phases: extrusion of zygotic embryos, formation of somatic embryos, and multiplication of embryogenic tissue into a culture.

The results from 1990 initiation studies (1991 Annual Forest Biology Review) indicated that DCR medium was superior for initiation. While callus extrusion occurred with other media, calli failed to proliferate unless they were transferred to DCR medium. Extrusion occurred in the absence of plant hormones but was apparently enhanced at high 2,4-D levels. The addition of a cytokinin (BA) stimulated extrusion at low 2,4-D levels when the ratio of 2,4-D to BA was 2:1. However, only 8 % of the megagametophytes extruded embryos. Most of these became brown and died and only 2-3% of the explants initiated cultures. Many of the cultures which proliferated did so on DCR medium containing 1.1 mg/l 2,4-D without cytokinin. Preliminary findings also indicated that zygotic embryos could be staged for development and on a 1-9 scale stages 2-4 were the most likely to initiate a culture.

Research during 1991 (1992 Annual Forest Biology Review) confirmed the effect of embryo stage with stage 2-3 pre-cotyledonary embryos showing the highest initiation rates. The addition of a cytokinin did not stimulate culture initiation. Maltose was better than sucrose for the production of embryogenic lines, and cold-sterilized sugars gave the best results. Initiated lines grew well on a medium containing a low auxin level and growth was best on maltose vs. sucrose.

In the summer of 1993 we began to explore the use of activated carbon to improve initiation rates in loblolly pine. Activated carbon (AC) had been reported to help initiate somatic embryo cultures in Douglas-fir (1) and radiata pine (2). In order to study the initiation process we decided to view it as a three part process. First extrusion of zygotic embryos from the ovule, then cleavage polyembryony of the zygotic embryo(s) present in the ovule (formation of the first somatic embryos), and then multiplication of the somatic embryos to form a small mass of embryogenic tissue suitable for transfer and subsequent maintenance.

Since the literature on the adsorption of plant hormones by AC often shows 95-99% adsorption of 2,4-D we decided to increase hormones by 100-200 times when combined with 2.5 g/l AC. Experiments showed that this combination of AC and hormones consistently increased extrusion from near 10% to 30-40%. Figure 1 shows extrusion results obtained in 1993 (presented in Forest Biology PAC Meeting - October 4-6, 1993). Over the next 1 1/2 years we learned that although extrusion was consistently increased by AC, initiation rates often remained below 1%. In 1994 we obtained no more than 18 initiations from more than 4100 explants (0.4%). Of these only 7 of the 18 cultures were maintained for at least 6 months. Our 1994 experiments focused on attempting to understand why initiation was inhibited in the presence of AC. Experiments presented in the March 1995 PAC Report demonstrated that AC adsorbed significant amounts of zinc and copper ions. With greater than 90% adsorption of copper, embryogenic cultures likely became deficient for this ion. With the addition of copper and zinc to compensate for adsorption, growth of somatic embryo explants from cultures already initiated was returned to normal in the presence of AC. However, new culture initiation rates still did not improve. A further inhibitory factor appeared to be present. During fall 1994 experiments were also conducted using somatic embryos to compare growth on initiation medium containing 2,4-D vs. NAA each at 75, 110, or 220 ppm combined with 2.5 g/l AC. Somatic embryo colony size showed statistically significant increases in size with NAA at 110 ppm vs. 110 or 220 ppm 2,4-D. Also, colony size decreased as 2,4-D increased. This data suggested that we may have too much auxin present in the initiation medium for optimal

somatic embryo growth. Also, it raised the question whether NAA was a better auxin for initiation and embryo growth than 2,4-D.

Since AC is known to adsorb 2,4-D, early on we desired information on actual free levels of 2,4-D available during initiation. We did not know if too little or too much 2,4-D was actually available to explants during the initiation process. Masters candidate Andrew Toering chose this problem for his A-190 Masters Thesis project. Mr. Toering's project began in Winter 1994 and was completed in Spring 1995. His results and findings are described in the following section of this Spring 1996 PAC Report. Mr. Toering's findings indicated that medium 201 (described in previous PAC reports with 2.5 g/l AC and 220 ppm 2,4-D) contained 12-17 ppm available 2,4-D during much of the initiation period. This amount of 2,4-D is 3-16 times greater than the 1-5 ppm 2,4-D often found in conifer initiation media. It is likely that this high concentration of 2,4-D is inhibitory to somatic embryo growth over time. Mr. Toering's findings suggested two approaches to decrease available 2,4-D in the presence of charcoal initiation media. 1) Lower 2,4-D concentrations from 220 to approximately 110 ppm in combination with 2.5 g/l AC. 2) Greatly lower AC levels and combine with a standard or slightly raised hormone combination.

An experiment was conducted in late Winter 1995 using Brazil cones from Westvaco. Charcoal was varied from 0 to 500 mg/l with 4 g/l Gelrite as a gelling agent. Base hormone levels of 2 ppm NAA, 0.45 ppm BAP, and 0.43 ppm Kinetin were used. We saw the greatest levels of extrusion and a few initiations when AC was present at 50-100 mg/l. Out of approximately 1800 Winter 1995 explants only six (0.3%) initiated small masses of embryos. Of these only two cultures survived after six months.

In June 1995 we became aware of a patent issued in late May, 1995 to Westvaco Co. for the use of low levels of gelling agents to improve pine initiation of embryogenic cultures. This patent suggested to us that our use of 4 g/l of Gelrite in initiation media might be inhibiting initiation rates. We decided to repeat the charcoal variation experiment using 2 g/l Gelrite (instead of the 4 g/l used earlier) as our first Summer 1995 experiment (Experiment 883). In addition, a high charcoal treatment with the suggested AC 2,4-D levels (2.5 g/l / 110 ppm) from Mr. Toering's work was added. Media composition for treatments in Experiment 883 are shown in Table 1. Figures 2 and 3 show the % Extrusion and % Initiation (9 week evaluations) respectively obtained for three genotypes. While statistically significant differences were not observed between individual treatments, a trend was evident for improved extrusion and initiation with lower levels of AC. In addition, medium 505 (Table 1) produced 3-10% initiation across three genotypes. Enough initiations occurred in the experimental treatments to statistically evaluate individual treatments.

Next we decided to repeat the Westvaco protocol in a 2x2x2 factorial arrangement to determine if we should license this patent to go forward in our research. Two salts were tested, DCR as described in the Westvaco patent and 1/2 P6 salts which we now use in all of our loblolly pine initiation, maintenance and development media. We also compared sucrose and maltose as two initiation sugars. The third factor was initiation medium osmolality with osmolality at approximately 145 mmol/kg or increased to 240 mmol/kg by adding 2% myo-inositol. All media contained 1 g/l Gelrite as recommended in the Westvaco Patent. No AC was added to media in this experiment (Experiment 884). The media composition for treatments in Experiment 884 are shown

in Table 2. Extrusion and initiation percentages are shown in Figures 4 and 5. On average DCR produced 22% extrusion while 1/2 P6 produced 27% extrusion. While not significant, improved extrusion on 1/2 P6 vs. DCR was in line with earlier experiments. Maltose (28% extrusion) caused significantly more extrusion than did sucrose (21% extrusion), $P = 0.07$. Differences in osmolality did not cause different extrusion rates. For initiation (9 week evaluations), all factors were non significant (Figure 6). Across all treatments initiation averaged 2.6%.

With the positive effects evident for low levels of AC combined with 2 g/l Gelrite, it was determined that an experiment should be run holding AC constant at 50 mg/l and varying Gelrite from 0 to 4 g/l. In this experiment (Experiment 887) all media contained the same composition as medium 505 in Table 1 except the gelling agent concentration was varied. A control medium with 2 g/l Gelrite but no AC was also included (medium 504). Final Extrusion % and Initiation % are shown in Figures 6 and 7 respectively. Extrusion % and initiation % increased as the gelling agent increased from none to 2 g/l. Peak extrusion and initiation occurred at 2 g/l (medium 505) and then decreased as Gelrite was increased to 4 g/l. These observations fit with previous data and show the deleterious effect that high Gelrite levels likely had on our earlier experiments.

An additional experiment combined 50 mg/l AC with 1.6 g/l Gelrite. Four sugar combinations (autoclaved sucrose, autoclaved maltose, autoclaved glucose, and filter-sterilized maltose) were each combined with two levels of osmolality (145 mmol/kg or 240 mmol/kg) in a 4 x 2 factorial arrangement (Experiment 888). Osmolality was adjusted with 2% myo-inositol in the higher level treatment. Percentages for Extrusion and Initiation were again high amongst all treatments (Figures 8 and 9). Although glucose provided the highest initiation % across three genotypes (25%) differences were not significant for sugars. Differences were nearly significant for osmolality ($P = 0.13$) with an average 19% initiation in the low osmolality treatment and 25% in the raised osmolality treatments. These results support our previous tentative conclusion that raised osmolality increased initiation rates.

Table 5 shows a summary of initiations for all media and all 1/2 sib families used during Summer 1995 initiation experiments. Cones were collected from eighteen families. Sixteen families contained early zygotic embryos at stages 2-4 suitable for initiation experiments. One family contained embryos that were too early or shriveled depending on the date of collection. Another family contained embryos at stages that were too late for experimentation. All sixteen families were able to initiate cultures with initiation rates ranging from 1 to 16%. Table 5 shows the total number of explants for each family, the percentage of initiation over all media tested, and culture survival after approximately six months. It is interesting to note that some families showed high levels of loss of cultures over time. Overall 6.8% initiation occurred across all families and 22% of the cultures initiated survived after six months. Obviously significant improvement in culture maintenance is necessary. Culture survival of 50% has been added to our future goals and will become a focus area for 1996 research.

Table 6 shows a summary of initiation responses for the best two media used across 10 families: medium 505 (50 mg /l AC /2 g/l Gelrite) and medium 539 (50 mg /l AC /1.6 g/l Gelrite). Across the 10 genotypes in which media 505 or 539 were tested, initiation averaged 16%. All ten families were able to initiate cultures with initiation rates ranging from 3-33%. In addition, some isolated

embryo explants also initiated cultures. This observation may provide hope for obtaining future cultures from dominant embryos within an ovule. The observations of 16% initiation across many genotypes show much promise for the future. In addition, we feel that further improvements are obtainable with minor changes in the medium. Culture survival also showed 20% over six months.

References

Pullman, G. S. and P. K. Gupta. 1994. United States Patent #5294549. Method for reproducing conifers by somatic embryogenesis using mixed growth hormones for embryo culture. Issued March 15, 1994.

Smith, D. R. A. P. Singh, and L. Wilton. 1985. Zygotic embryos of Pinus radiata in vivo and in vitro. In. Proc. Third Meeting International Conifer Tissue Culture Work Group 12-16 August, 1985, Rotorua, New Zealand. pp 21.

RP883	Media (mg/l)					
Components	504	505	506	507	508	509
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
$Ca(NO_3)_2 \cdot 4H_2O$	236.2	236.2	236.2	236.2	236.2	236.2
$MgSO_4 \cdot 7H_2O$	246.5	246.5	246.5	246.5	246.5	246.5
$Mg(NO_3)_2 \cdot 6H_2O$	256.5	256.5	256.5	256.5	256.5	256.5
$MgCl_2 \cdot 6H_2O$	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
$MnSO_4 \cdot H_2O$	10.5	10.5	10.5	10.5	10.5	10.5
$ZnSO_4 \cdot 7H_2O$	14.4	14.688	14.976	15.84	17.28	28.8
$Na_2MoO_4 \cdot 2H_2O$	0.125	0.125	0.125	0.125	0.125	0.125
$CuSO_4 \cdot 5H_2O$	0.125	0.1725	0.22	0.3625	0.6	2.375
$CoCl_2 \cdot 6H_2O$	0.125	0.125	0.125	0.125	0.125	0.125
$FeSO_4 \cdot 7H_2O$	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	--	--	--	--	--	110
NAA	2.0	2.0	2.0	2.0	2.0	--
BAP	0.45	0.45	0.45	0.45	0.45	45
Kinetin	0.43	0.43	0.43	0.43	0.43	43
Activated Charcoal	--	50	100	250	500	2,500
Gelrite	2,000	2,000	2,000	2,000	2,000	2,000
pH	5.7	5.7	5.7	5.7	5.7	5.7

Table 1. Media composition for treatments used in experiment 883.

RP884	Media (mg/l)							
Components	514	515	516	517	510	511	512	513
NH_4NO_3	200.0	200.0	200.0	200.0	400	400	400	400
KNO_3	909.9	909.9	909.9	909.9	340	340	340	340
KH_2PO_4	136.1	136.1	136.1	136.1	170	170	170	170
$Ca(NO_3)_2 \cdot 4H_2O$	236.2	236.2	236.2	236.2	556	556	556	556
$MgSO_4 \cdot 7H_2O$	246.5	246.5	246.5	246.5	370	370	370	370
$Mg(NO_3)_2 \cdot 6H_2O$	256.5	256.5	256.5	256.5	--	--	--	--
$MgCl_2 \cdot 6H_2O$	101.7	101.7	101.7	101.7	--	--	--	--
$CaCl_2 \cdot 2H_2O$	--	--	--	--	85	85	85	85
KI	4.15	4.15	4.15	4.15	0.83	0.83	0.83	0.83
H_3BO_3	15.5	15.5	15.5	15.5	6.2	6.2	6.2	6.2
$MnSO_4 \cdot H_2O$	10.5	10.5	10.5	10.5	22.3	22.3	22.3	22.3
$ZnSO_4 \cdot 7H_2O$	14.4	14.4	14.4	14.4	8.6	8.6	8.6	8.6
$Na_2MoO_4 \cdot 2H_2O$	0.125	0.125	0.125	0.125	0.25	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.125	0.125	0.125	0.125	0.25	0.25	0.25	0.25
$CoCl_2 \cdot 6H_2O$	0.125	0.125	0.125	0.125	0.025	0.025	0.025	0.025
$NiCl_2 \cdot 6H_2O$	--	--	--	--	0.025	0.025	0.025	0.025
$FeSO_4 \cdot 7H_2O$	13.9	13.9	13.9	13.9	27.8	27.8	27.8	27.8
Na_2EDTA	18.65	18.65	18.65	18.65	37.3	37.3	37.3	37.3
Maltose	--	30,000	--	15,000	--	30,000	--	15,000
Sucrose	30,000	--	15,000	--	30,000	--	15,000	--
myo-Inositol	500	500	20,000	20,000	500	500	20,000	20.000
Casamino acids	500	500	500	500	500	500	500	500
L-Glutamine	450	450	450	450	250	250	250	250
Thiamine•HCl	1.0	1.0	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	0.5	0.5
Nicotinic acid	0.5	0.5	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	2.0	2.0
2,4-D	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
BAP	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Gelrite	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1.000
pH	5.7	5.7	5.7	5.7	5.7	5.7	5.7	5.7

Table 2. Media composition for treatments used in experiment 884.

RP887	Media (mg/l)						
Components	504	505	531	532	533	534	535
NH_4NO_3	200.0	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	909.9
KH_2PO_4	136.1	136.1	136.1	136.1	136.1	136.1	136.1
$Ca(NO_3)_2 \cdot 4H_2O$	236.2	236.2	236.2	236.2	236.2	236.2	236.2
$MgSO_4 \cdot 7H_2O$	246.5	246.5	246.5	246.5	246.5	246.5	246.5
$Mg(NO_3)_2 \cdot 6H_2O$	256.5	256.5	256.5	256.5	256.5	256.5	256.5
$MgCl_2 \cdot 6H_2O$	101.7	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	4.15
H_3BO_3	15.5	15.5	15.5	15.5	15.5	15.5	15.5
$MnSO_4 \cdot H_2O$	10.5	10.5	10.5	10.5	10.5	10.5	10.5
$ZnSO_4 \cdot 7H_2O$	14.4	14.688	14.688	14.688	14.688	14.688	14.688
$Na_2MoO_4 \cdot 2H_2O$	0.125	0.125	0.125	0.125	0.125	0.125	0.125
$CuSO_4 \cdot 5H_2O$	0.125	0.1725	0.1725	0.1725	0.1725	0.1725	0.1725
$CoCl_2 \cdot 6H_2O$	0.125	0.125	0.125	0.125	0.125	0.125	0.125
$FeSO_4 \cdot 7H_2O$	13.9	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	18.65
Maltose	15,000	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	20,000
Casamino acids	500	500	500	500	500	500	500
L-Glutamine	450	450	450	450	450	450	450
Thiamine•HCl	1.0	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	0.5
Nicotinic acid	0.5	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	2.0
2,4-D	--	--	--	--	--	--	--
NAA	2.0	2.0	2.0	2.0	2.0	2.0	2.0
BAP	0.45	0.45	0.45	0.45	0.45	0.45	45
Kinetin	0.43	0.43	0.43	0.43	0.43	0.43	43
Activated Charcoal	--	50	50	50	50	50	50
Gelrite	2,000	2,000	--	250	400	1,000	4,000
pH	5.7	5.7	5.7	5.7	5.7	5.7	5.7

Table 3. Media composition for treatments used in experiment 887.

RP888 Components	Media (mg/l)							
	536	537	538	539	540	541	542	543
NH_4NO_3	200.0	200.0	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	909.9	909.9
KH_2PO_4	136.1	136.1	136.1	136.1	136.1	136.1	136.1	136.1
$Ca(NO_3)_2 \cdot 4H_2O$	236.2	236.2	236.2	236.2	236.2	236.2	236.2	236.2
$MgSO_4 \cdot 7H_2O$	246.5	246.5	246.5	246.5	246.5	246.5	246.5	246.5
$Mg(NO_3)_2 \cdot 6H_2O$	256.5	256.5	256.5	256.5	256.5	256.5	256.5	256.5
$MgCl_2 \cdot 6H_2O$	101.7	101.7	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	4.15	4.15
H_3BO_3	15.5	15.5	15.5	15.5	15.5	15.5	15.5	15.5
$MnSO_4 \cdot H_2O$	10.5	10.5	10.5	10.5	10.5	10.5	10.5	10.5
$ZnSO_4 \cdot 7H_2O$	14.688	14.688	14.688	14.688	14.688	14.688	14.688	14.688
$Na_2MoO_4 \cdot 2H_2O$	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
$CuSO_4 \cdot 5H_2O$	0.1725	0.1725	0.1725	0.1725	0.1725	0.1725	0.1725	0.1725
$CoCl_2 \cdot 6H_2O$	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
$FeSO_4 \cdot 7H_2O$	13.9	13.9	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	18.65	18.65
Sucrose	30,000	15,000	--	--	--	--	--	--
Maltose	--	--	30,000	15,000	--	--	30,000*	15,000*
Glucose	--	--	--	--	15,000	15,000	--	--
myo-Inositol	500	20,000	500	20,000	500	20,000	500	20,000
Casamino acids	500	500	500	500	500	500	500	500
L-Glutamine	450	450	450	450	450	450	450	450
Thiamine•HCl	1.0	1.0	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	0.5	0.5
Nicotinic acid	0.5	0.5	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	2.0	2.0
NAA	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
BAP	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Kinetin	0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43
Activated Charcoal	50	50	50	50	50	50	50	50
Gelrite	1,600	1,600	1,600	1,600	1,600	1,600	1,600	1,600
pH	5.7	5.7	5.7	5.7	5.7	5.7	5.7	5.7

*This treatment uses filter sterilized maltose added to media after autoclaving

Table 4. Media composition for treatments used in experiment 888.

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Table 5. Overall initiation rates for all media tested and six month culture survival across sixteen 1/2 sib families during Summer, 1995.

Clone	# Initiations / Total	% Initiation	# Survived / Total Initiations	% Survial of Initiations
BC-1	5 / 480	1.0	3 / 5	60
BC-2	15 / 420	3.6	0 / 15	0
BC-3	37 / 469	7.9	3 / 37	8.1
BC-5	15 / 240	6.2	1 / 15	6.7
BC-8	Embryos too early or shriveled.	-	-	
BC-9	27 / 420	6.4	11 / 27	41
UC5-1036	87 / 529	16.4	25 / 87	29
UC7-1051	5 / 240	2.0	3 / 5	60
UC7-1037	7 / 210	3.3	0 / 7	0
UC10-5	8 / 420	1.9	0 / 8	0
UC10-33	28 / 420	6.7	8 / 28	29
UC10-1027	45 / 450	10.0	5 / 45	11
UC11-1055	4 / 240	1.7	0 / 4	0
WV-F2	87 / 714	12.1	21 / 87	24
WV-G2	Stages too late.	-	-	-
WV-H2	23 / 240	9.6	11 / 23	48
WV-I2	17 / 450	3.8	4 / 17	24
WV-J2	26 / 480	5.4	2 / 26	7.7
Overall Totals	**436 / 6422**	**6.8**	**97 / 436**	**22**

Table 6. Initiation rates on medium 505 and 539 and six month culture survival for ten 1/2 sib families collected during Summer, 1995.

Clone	# Initiations / Total	% Initiation	# Survived / Total Initiations	% Survial of Initiations
BC-2	3 / 30	10	0 / 3	0
BC-3	18 / 109	17	3 / 18	17
BC-9	10 / 60	25	4 / 10	40
UC5-1036	25 / 79	32	6 / 25	24
UC7-1037	3 / 30	10	0 / 3	0
UC10-5	1 / 30	3.3	0 / 1	0
UC10-33	7 / 60	12	2 / 7	29
UC10-1027	10 / 30	33	0 / 10	0
WV-F2	12 / 114	11	4 / 12	33
WV-I2	9 / 60	15	1 / 9	11
Overall Totals	**98 / 602**	**16**	**20 / 98**	**20**

% Extrusion vs Treatment
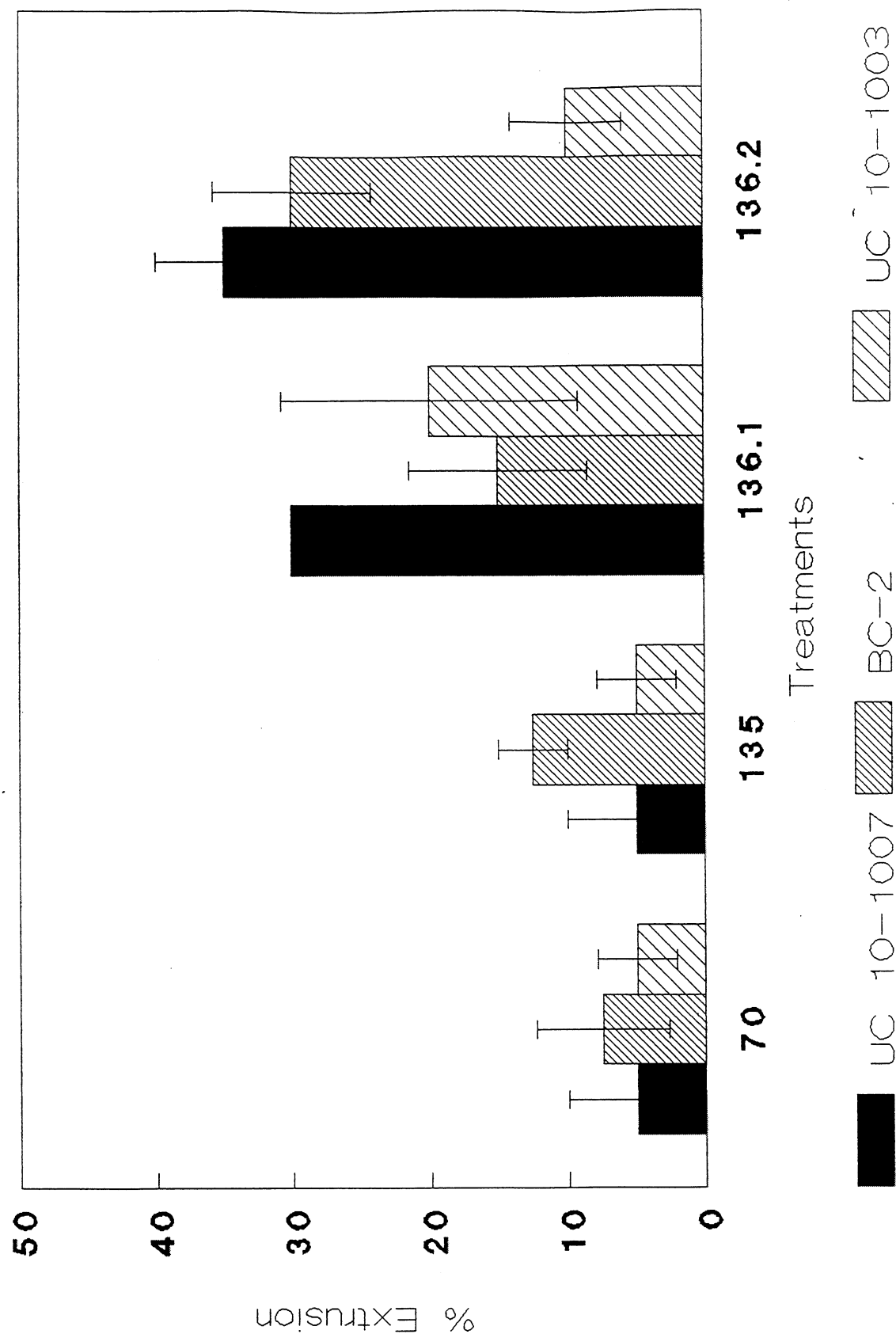
Figure 1. Effect of clear (without charcoal) gelrite (70) or agar (135) medium and media prepared with charcoal (136.1) or with a transfer to the same charcoal medium after 10 days (136.2) on embryo extrusion from cultured megagametophytes of Loblolly Pine.
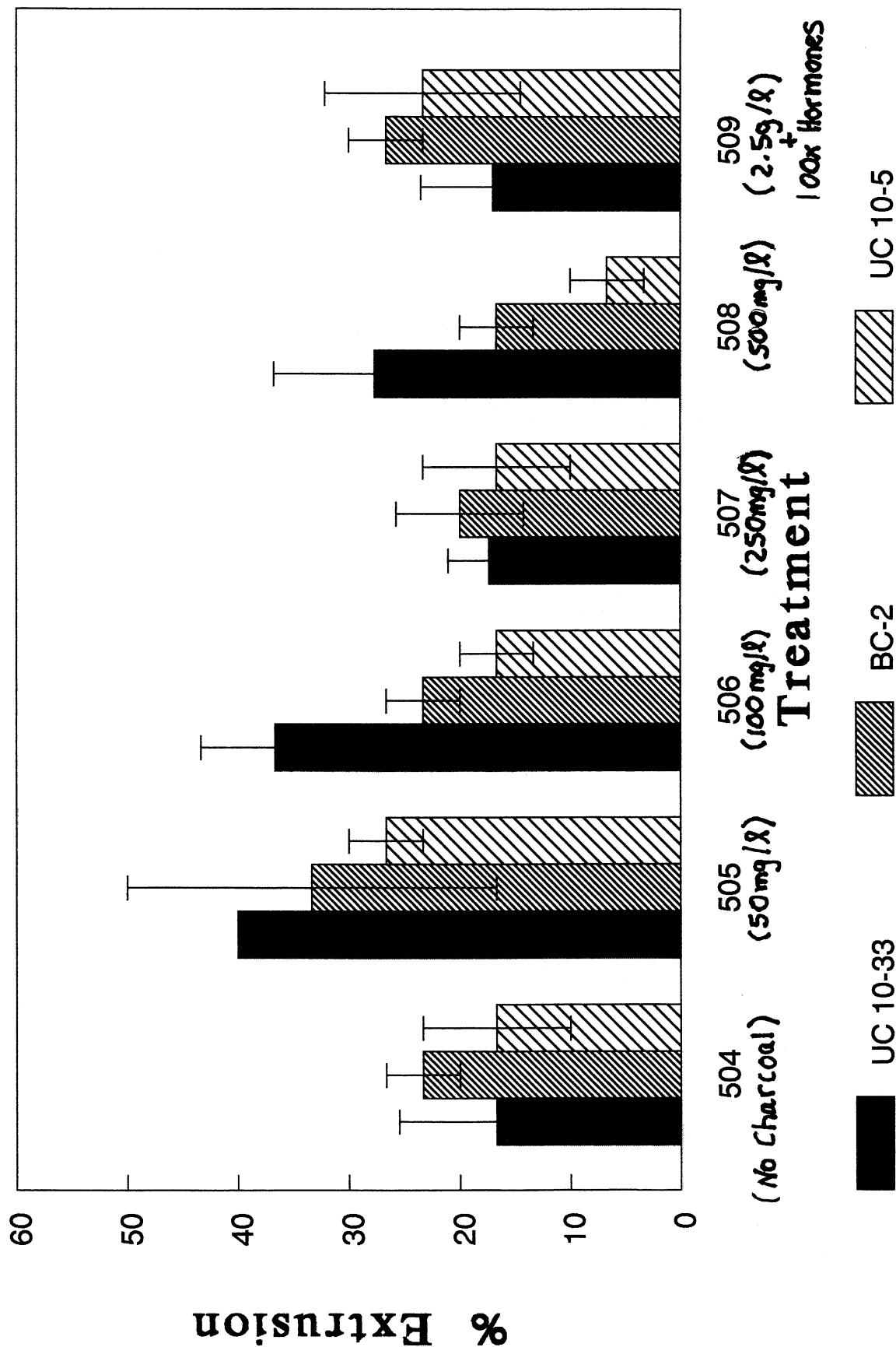
Experiment 883
% Extrusion

Figure 2. Effect of varying levels of activated charcoal on extrusion from cultured megagametophytes of loblolly pine (Experiment 883).
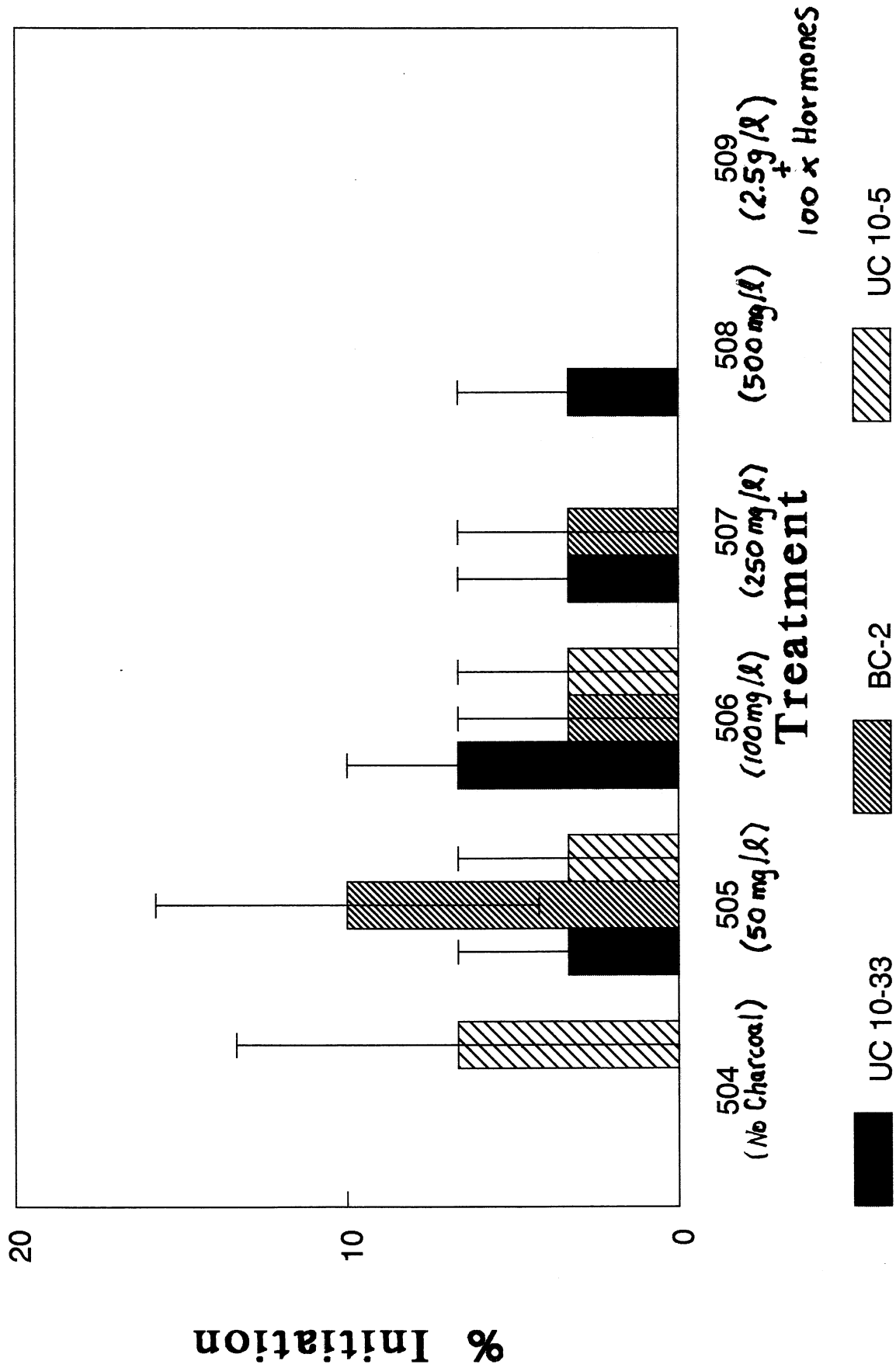
21

Experiment 883
% Initiation

Figure 3. Effect of varying levels of activated charcoal on initiation of somatic embryos from cultured megagametophytes of loblolly pine (Experiment 883).
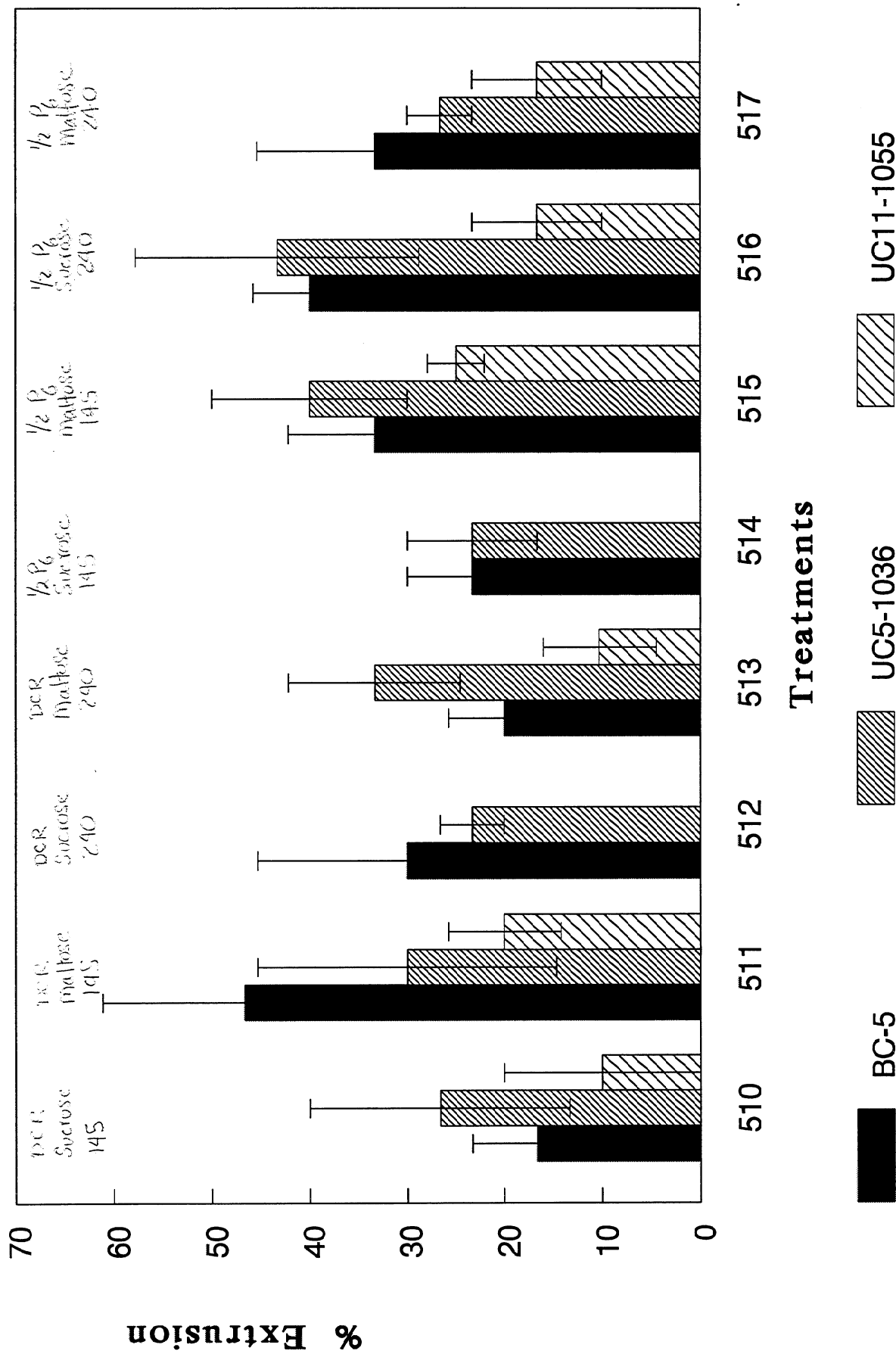
Experiment 884
% Extrusion

Figure 4. Effect of varying salts, sugar type, and osmolality on extrusion from cultured megagametophytes of loblolly pine (Experiment 884). All treatments were gelled with 1 g/l Gelrite.
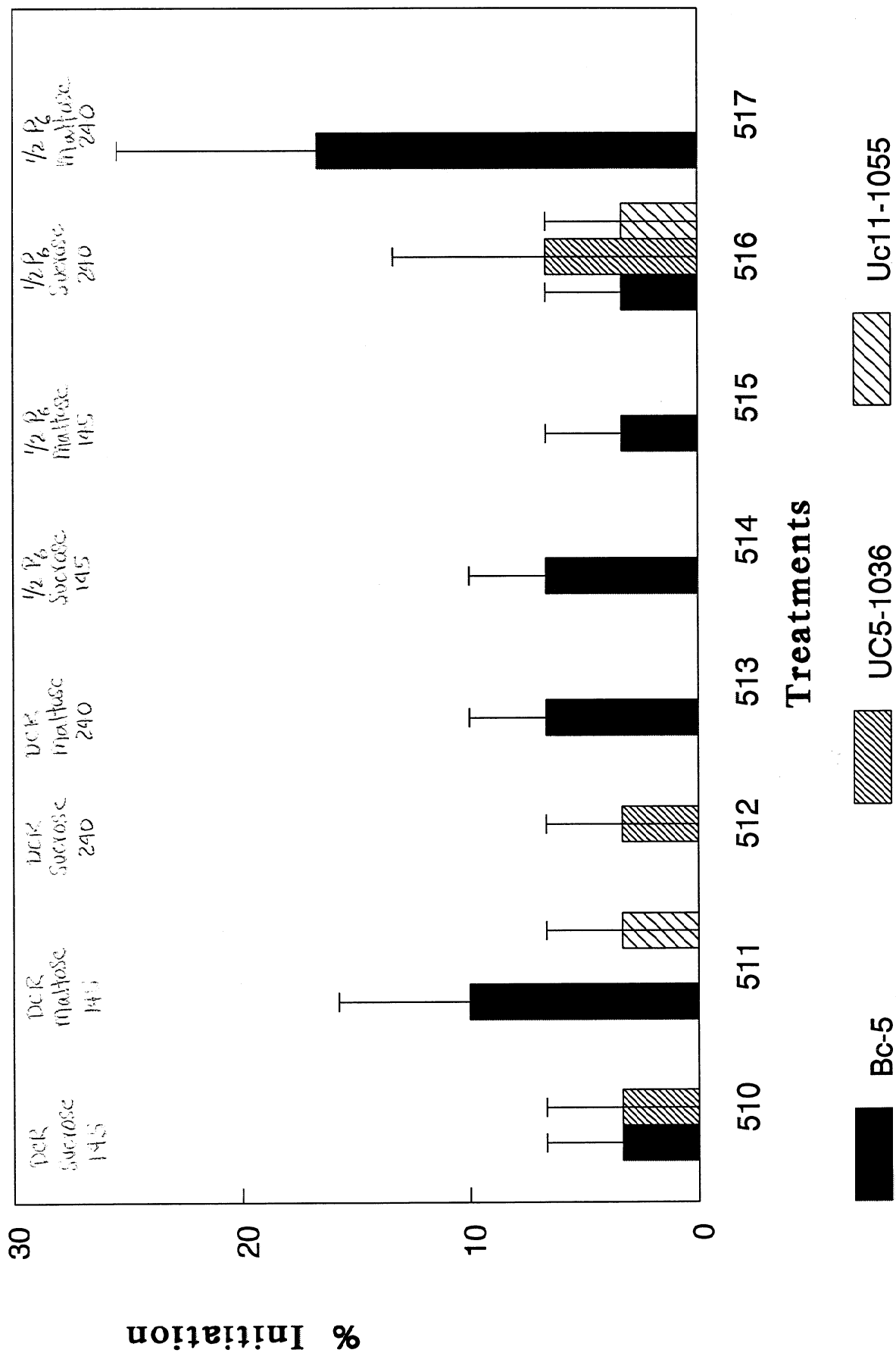
23

Experiment 884
% Initiation

Figure 5. Effect of varying salts, sugar type, and osmolality on initiation of somatic embryos from cultured megagametophytes of loblolly pine (Experiment 884). All treatments were gelled with 1 g/l Gelrite.

Experiment 887
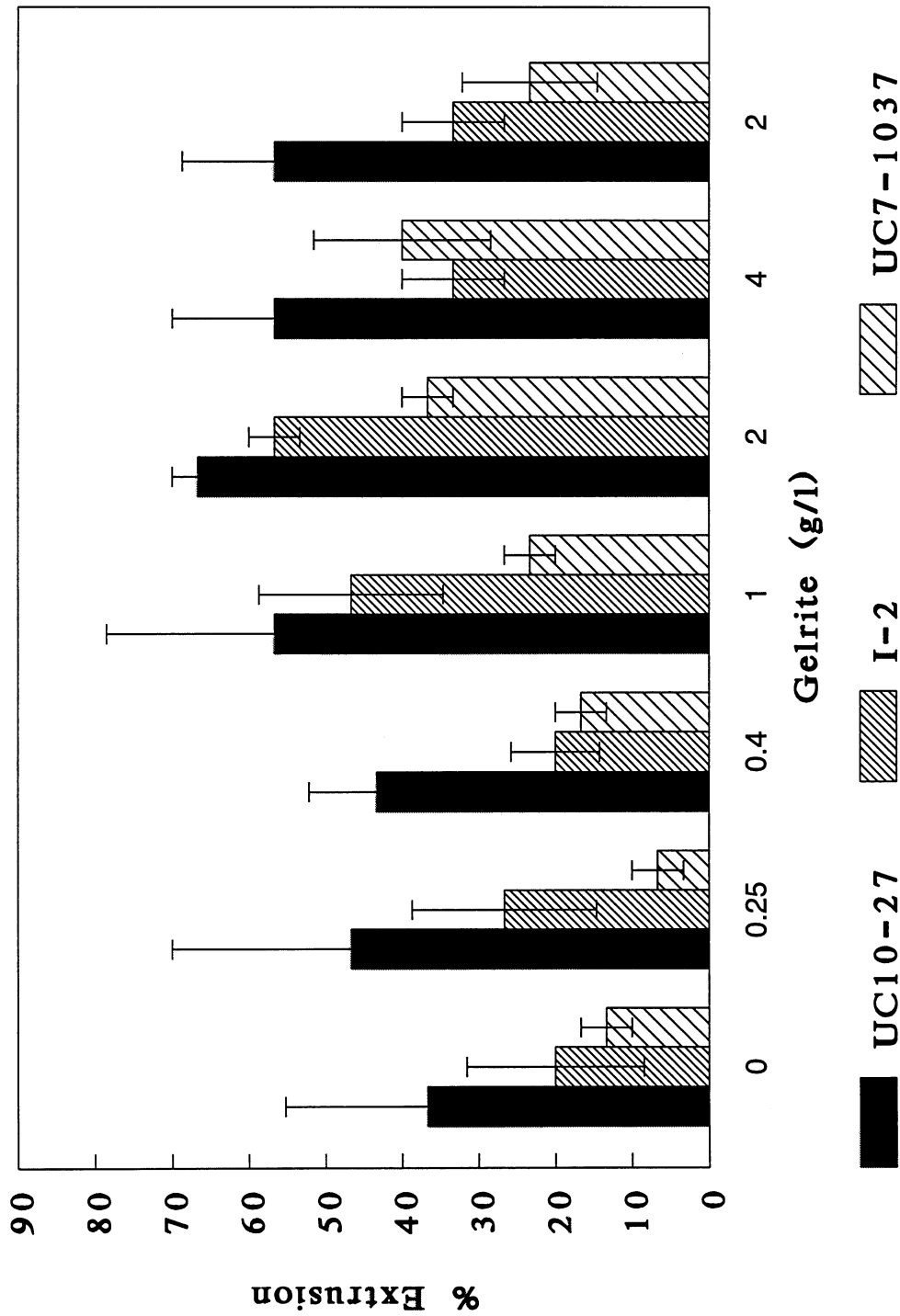## % Extrusion vs. Gelrite

Figure 6. Effect of varying Gelrite concentration on extrusion from cultured megagametophytes of loblolly pine (Experiment 887). The last set of bars shown is for medium 504 which contained 2 g/l Gelrite and no activated carbon (AC). The remaining sets of bars shown are for media varying in Gelrite concentration with 50 mg/l AC.

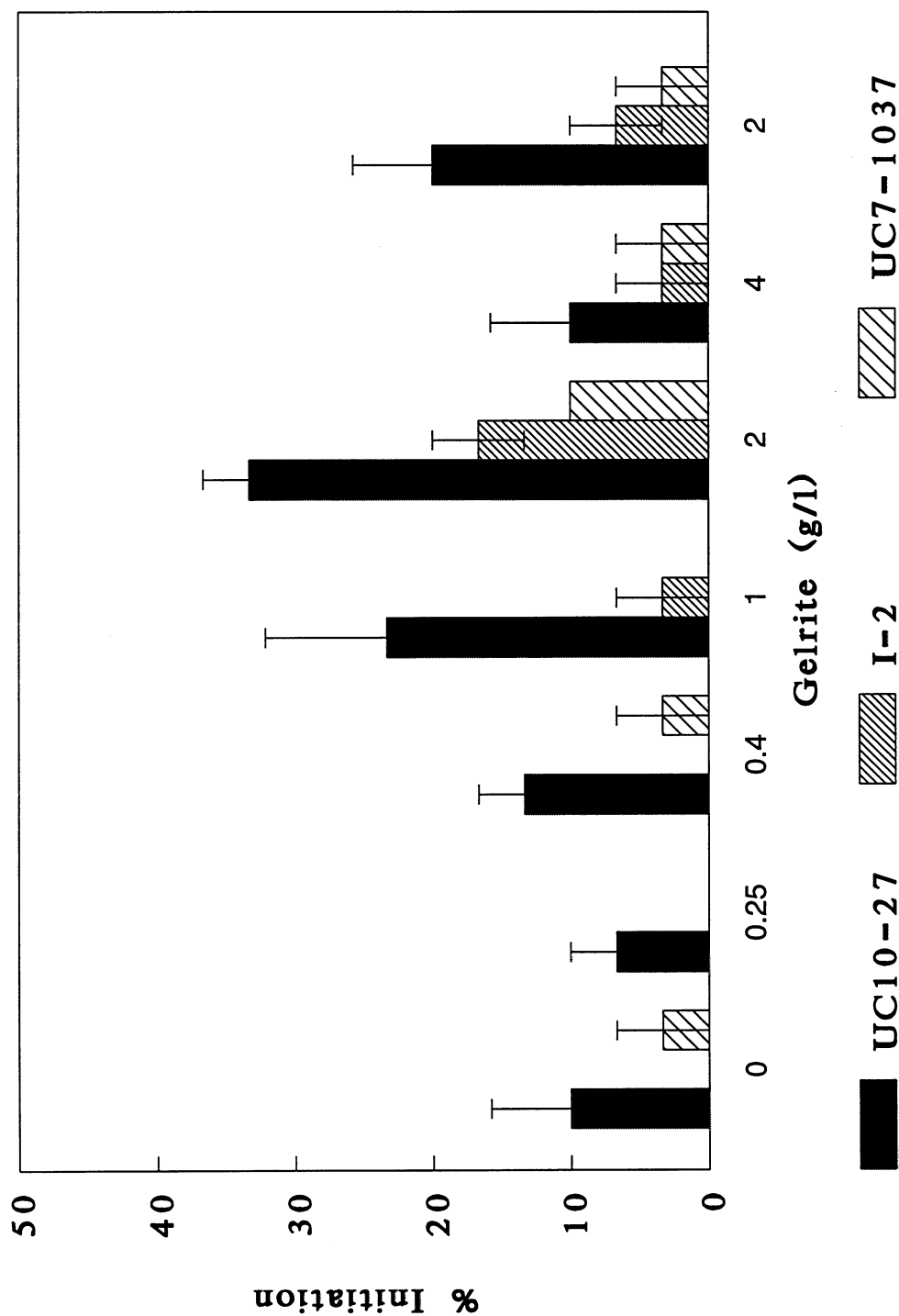# Experiment 887
% Initiation vs. Gelrite

Figure 7. Effect of varying gelrite concentration on initiation of somatic embryos from cultured megagametophytes of loblolly pine (Experiment 887).). The last set of bars shown is for medium 504 which contained 2 g/l Gelrite and no activated carbon (AC). The remaining sets of bars shown are for media varying in Gelrite concentration with 50 mg/l AC.

Experiment 888
% Extrusion
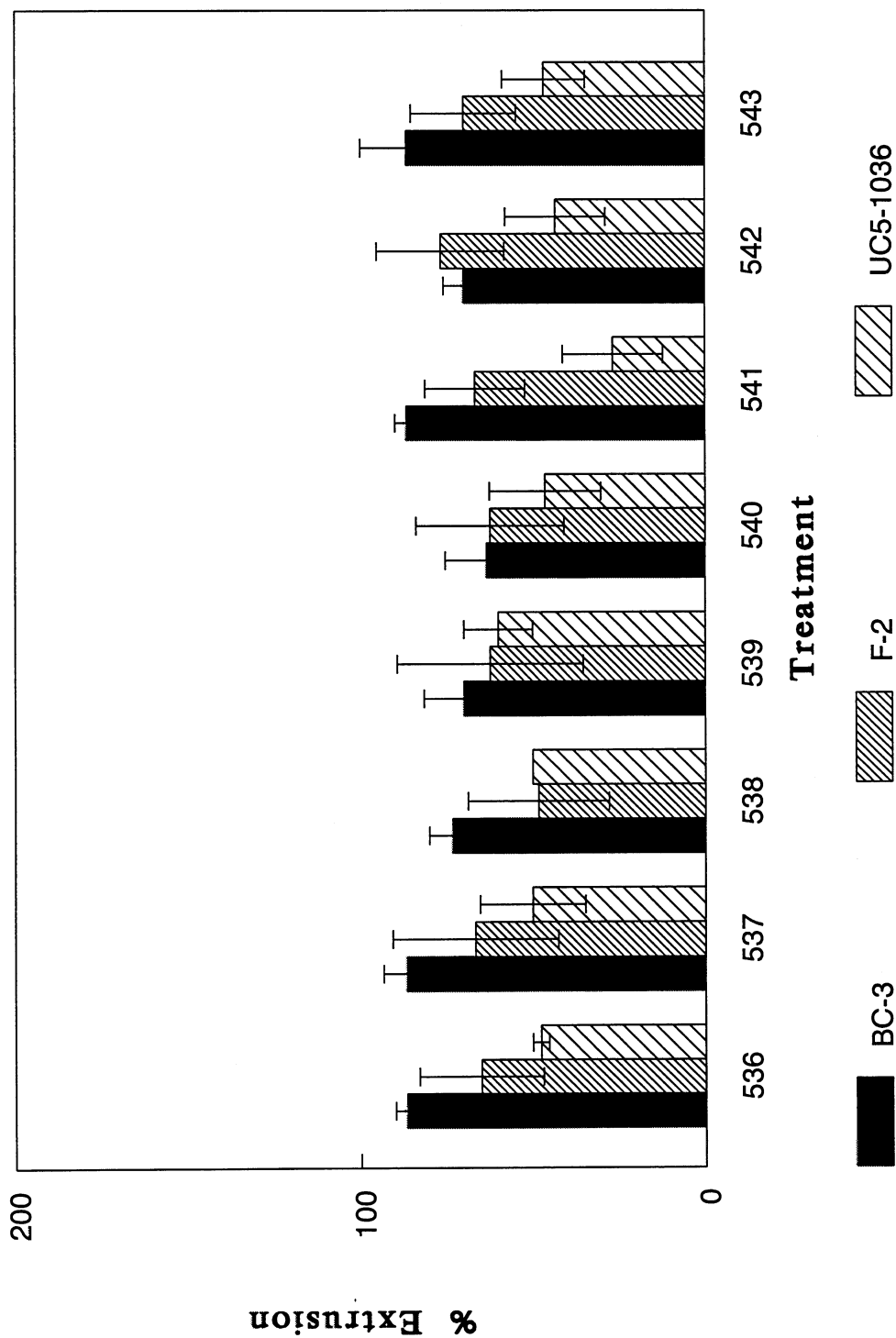
Figure 8. Effect of varying sugar type, sterilization method, and osmolality on extrusion from cultured megagametophytes of loblolly pine (Experiment 888). All treatments were gelled with 1.6 g/l Gelrite and contained 50 mg/l activated carbon.

Figure 9. Effect of varying sugar type, sterilization method, and osmolality on initiation of somatic embryos from cultured megagametophytes of loblolly pine (Experiment 888). All treatments were gelled with 1.6 g/l Gelrite and contained 50 mg/l activated carbon.

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MASS CLONAL PROPAGATION OF
IMPROVED CONIFERS - INITIATION

Examining the Relationship between 2,4-Dichlorophenoxyaccetic Acid and
Activated Carbon in Plant Tissue Culture Media

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - INITIATION
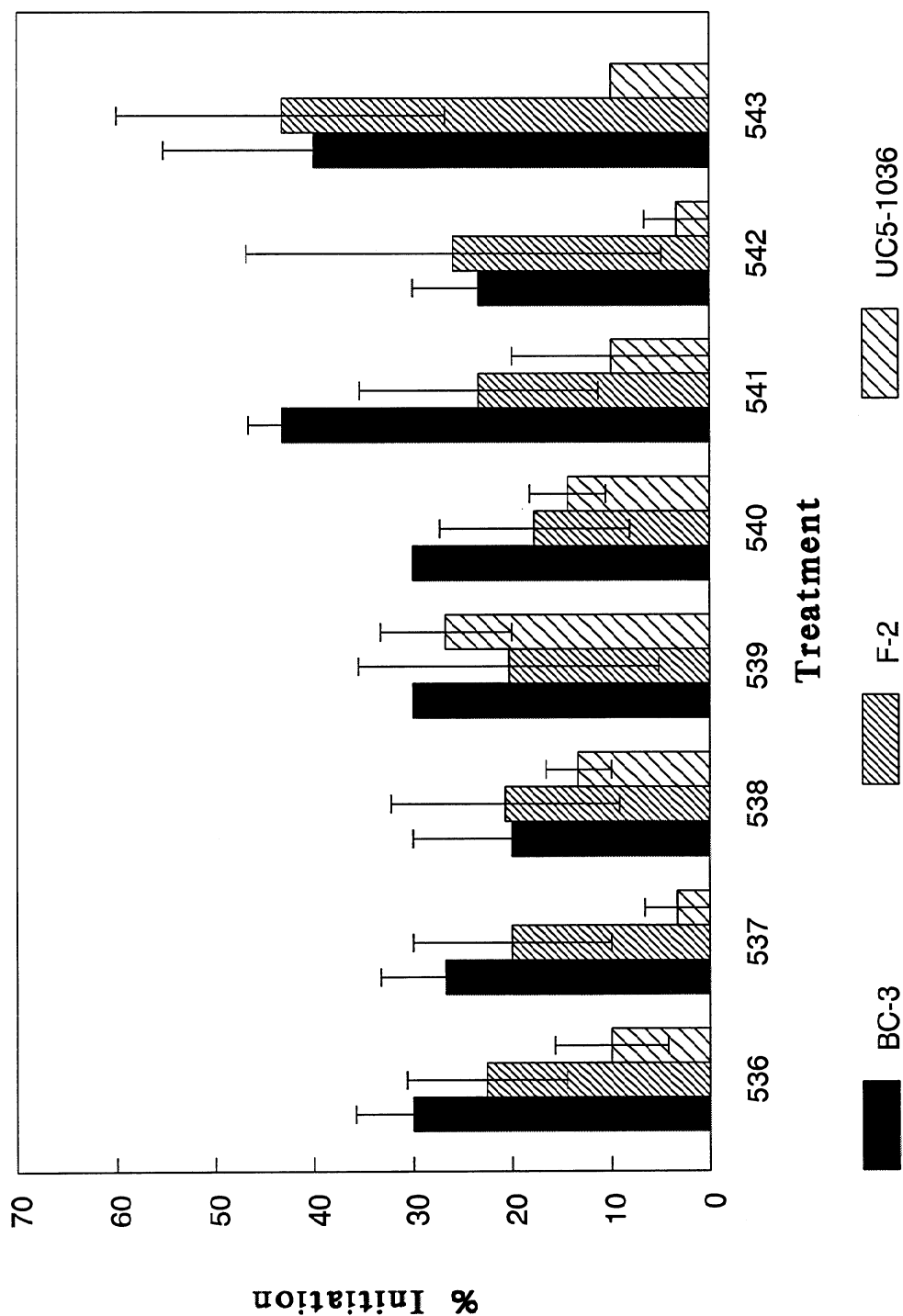
Examining the Relationship between 2,4-Dichlorophenoxyacetic Acid and Activated Carbon in Plant Tissue Culture Media

Andrew Toering
Gerald Pullman

Following is a summary of the final research report from the A-190 of Andrew Toering. For greater detail please see the original document submitted in partial fulfillment of the MS Degree. (Toering, Andrew J. Examining the relationship between 2,4-Dichlorophenoxyacetic acid and activated charcoal in plant tissue culture media. Institute of Paper Science and Technology, May 24, 1995. Pages 1-80.)

Abstract

The clonal propagation of high value forest trees through somatic embryogenesis has the potential to help meet future industry needs for uniform and high quality raw materials. Low culture initiation frequencies for loblolly pine (*Pinus taeda*) currently pose a limitation in work towards commercialization of this technology. Most work on somatic embryo culture initiation in loblolly pine reports success in the range of 1-5%. Activated carbon (AC) has been reported to improve many tissue culture systems including initiation in Douglas-fir (Forest Biology Fall 1993 PAC Report). To improve initiation frequencies in loblolly pine our program began exploring development of an AC-containing system. In order to better understand the availability of 2,4-D in initiation medium, research was begun to track media surface concentrations of free or available 2,4-D. Media containing 1/2 P6 salts, 1.5% maltose, 2% myo-inositol, case amino acids, glutamine, vitamins, and 0.4% Gelrite were modified to include 0.625 - 2.5 g/l of activated carbon (Sigma C-9157, acid washed) and 110 - 440 mg/l 2,4-D. Availability of 2,4-dichlorophenoxyacetic acid (2,4-D) in AC-containing medium was tracked by C^{14} labeled 2,4-D present in surface moisture absorbed onto filter paper.

High correlation coefficients were found for 1) available 2,4-D and AC concentrations and 2) for initial 2,4-D levels and resulting free 2,4-D. This model allows target levels of available 2,4-D to be met by adjustment of initial 2,4-D levels or medium AC content. The model also predicts that with our standard levels of 2,4-D (220 ppm) and 2.5 g/l AC, free 2,4-D is probably high for embryogenic tissue growth.

Introduction

Tissue culture techniques enable biologists and foresters to reproduce a large number of essentially identical individual genotypes (32). Newly developed techniques, like somatic embryogenesis have played increasingly important roles in commercial development of plants with specific desired characteristics (18). These techniques can be optimized to maximize the amount of healthy growth attainable in each species. Through essentially trial and error methodology, successful propagation techniques have been developed for many species.

One approach in the effort to optimize these methods which has consistently yielded beneficial results is the addition of activated carbon (AC) to the culture media (8,10,11,13,14,21,22,33,44). Although several experiments have been undertaken to observe the effects of AC on embryonic growth, a definitive model or mechanism has not yet been completely proven. Known primarily for its adsorptive properties in the purification of water (2,6,28,30), hypotheses generally have been generated that reflect the adsorptive characteristics in its role in tissue culture medium (10,13,23,33). Assuming this to be true, what remains to be discovered is the rate of adsorption, as well as what is truly being adsorbed, the growth hormones present in the system waste by-products due to growth mechanisms, or some combination of the two, or other combinations not yet considered.

Recently, more reports of growth hormones and activated carbon (AC) acting in a linked relationship have come to light. It already has been shown that AC has adsorption effects on some growth hormones, such as abscisic acid (ABA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (10,13,17,32), but a definitive model describing this relationship has not yet been published. Quantitatively describing these relationships is important in the method of embryogenesis and other plant tissue culture techniques, in that it would enable the researcher to control the growth of the desired tissue while maintaining target levels of plant hormones. This project sought to develop a definitive mathematical model for the rate of adsorption of 2,4-D in tissue culture media.

Materials and Methods

The basic methodology employed in this research project was essentially patterned after the methods of Gupta and Pullman (17); in that the plant growth regulator, 2,4-D, was doped with a radio-labeled portion of the PGR and placed in a medium containing AC. The corresponding relative decrease in the availability of the free 2,4-D was then followed using a scintillation counter. Key differences between the methods reported in this study and previous researchers are the preparation of the medium, and the collection of data. The media formulation is shown in Table 1.

A stock solution of 2,4-dichlorophenoxyacetic acid-carboxy-14C was prepared with some difficulty. 1.8 mg of labeled 2,4-D was introduced into 20 ml of 80:20 Ethanol/H_2O

carrier solvent as recommended. When time and agitation did not visually improve the dissolving process 1 ml of 1 N KOH was added. With further agitation the labeled 2,4-D dissolved. Two hundred μL aliquots of solution, containing 0.017 mg of labeled 2,4-D, were used to spike media prepared with 110, 220, or 440 mg/l of non-labeled 2,4-D. When tested with the scintillation counter, 10 mls of medium without AC was calculated to yield approximately 3.50×10^4 DPM.

Media components were divided into two parts while mixing, one portion containing AC and double-distilled water, and the other containing the balance of the media components dissolved in double-distilled water. The separate portions were autoclaved as such for 20 minutes at 121 C and 131 kPa. After cooling somewhat, all flasks were placed in a hot water bath maintained at 55-60 °C. Once the media components had come to bath temperature, glutamine was added to the flasks without AC. The flasks were then transferred to the radiation work area, where 200 μL of labeled 2,4-D solution was added to the non-charcoal containing solution in a portable sterile hood. The charcoal portion was then poured into the balance of the media components under mixing on a stirrer plate in the hood. This mixing marks time zero as the start of the sampling periods.

The media was allowed to mix for approximately 10 minutes before the plates were poured. Fifty plates were filled for each series with 10 ml of warm media dispersed as two 5 ml aliquots. Plates were poured in stacks of five. The plates were allowed to remain undisturbed for a minimum of 20 minutes so that the gelling process would not be disrupted. Once the plates had gelled, they were transferred stack by stack to holding containers, where they would remain for the duration of the experiment.

At each designated testing period, plates were selected from the top row of the stacks so as not to sample directly from plates poured at nearly the same moment. Using forceps, a piece of 4.7 cm Whatman GF/A filter paper was placed in contact with the surface of the media. After 30 seconds, the paper was removed and placed in a scintillation vial. On average, each filter paper absorbed 0.46 g of surface liquid medium without charcoal present. Once all of the selected plates had been sampled, 20 ml of scintillation cocktail was added to each vial. Vials were capped and manually agitated to insure wetting of the filter paper. The vials were then counted in a Beckman 3801 scintillation counter and results were obtained from an attached printer. Throughout the media preparation, dispensing, sample collection, and disposal process safety procedures were followed.

Once data for the control was obtained, it was necessary to describe it mathematically in order to effectively utilize the information as a baseline. This was accomplished through fitting a second degree polynomial to the data points. Once the equation had been finalized, data from the sampled plates could then be related back to the control, and then to each other. All of the results were normalized by dividing the sampled DPM by the polynomial describing the control at the given time period, and multiplying by 100 to obtain a percent value. This would later be multiplied by the initial concentration of 2,4-D for each series in order to obtain literal values. Thus, the relative percentages of

available 2,4-D were obtained for each of two data series. This then allowed the comparison between separate series. For ease of understanding, the percentage of available 2,4-D was converted into a part per million basis from the initial concentration in the media. The information was then compiled and plotted.

Results and Discussion

Two main series of data were obtained. The first series was for long-term adsorption and plates were sampled at 1, 3, 5, 7, 10, 15, 21, and 28 days from the point of mixing all of the components. The second series with shorter time periods was carried out after measuring the rapid 2,4-D adsorption seen in the long-term series. The second series sampled plates at 1, 6, 12, and 24 hours and after 2, 3, 5, and 7 days.

Controls for each time series were run without charcoal in order to compensate for changes in 2,4-D concentration over time not due to activated charcoal. Once the control data was obtained, it was necessary to describe it mathematically in order to effectively utilize the information as a baseline. This was accomplished through fitting a second degree polynomial to the data points. Control curves for the long-term and short-term series are shown in Figure 1. It is interesting to note that in both series available 2,4-D increased over time. Approximately half of this increase can be accounted for by evaporation of water from plates over time. Once the control equation had been finalized sampled plates could then be related back to the control, and then to each other. All of the results were normalized by dividing the sampled DPM by the polynomial describing the control at the given time period, and multiplying by 100 to obtain a percent value. The percent value was then multiplied by the initial 2,4-D concentration to obtain literal values. By doing so, the relative available 2,4-D levels were obtained for each time period in each series. (A more lengthy description of the data manipulations can be found in the original A-190 document.)

Prior to an in-depth analysis of the obtained results, some general formatting procedures need to be discussed. The line through the data points represents the second degree polynomial curve best fitting the data. The equation for the curve is shown along with the R^2 correlation coefficient. Curves were drawn based only on the measured data points and excluded the theoretical initial added 2,4-D level at zero time. The abbreviations LT and ST stand for the long-term and short-term series. The error bars represent the standard deviation based on the number of values contributing to that point.

The first series to be examined are the long-term series, starting with 2,4-D concentrations at 110 ppm evaluated at three levels of activated carbon, noted on the graphs found in Figure 2. At the lowest concentration of charcoal, 0.0625%, an equilibrium value of ~70 ppm is reached by 15 days. As the charcoal level is doubled to 0.125%, an equilibrium value of ~25 ppm was reached by the third day. The amount of available 2,4-D was decreased by 35% and an apparently steady state condition was reached within 72 hours. As the charcoal is again doubled, an equilibrium value of ~3

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ppm is reached within 5 days. This represents only 4% of the amount of 2,4-D available at the lowest charcoal concentration.

The next complete series to be examined contains two levels of AC (0.0625% and 0.25%) at an initial concentration of 440 ppm 2,4-D (Figure 3). At the lower AC concentration the results are difficult to accurately interpret due to a wide standard deviation associated with many of the data points and due to an apparent increase in the amount of available 2,4-D, surpassing the original concentration level. As for the flat line denoted by the results, it may show that at low AC/high 2,4-D concentrations, the charcoal is immediately saturated with 2,4-D. When the charcoal is increased by a factor of four, the expected sorption pattern is again observed, with an equilibrium value of 160 ppm reached by 15 days, which is 36% of the initial concentration. The portion of this media containing 0.125% AC and 440 ppm 2,4-D did not gel, or solidify, for unknown reasons, and this accounts for the missing third concentration.

The last series from the long-term classification again looks at three AC concentrations at 220 ppm initial 2,4-D (Figure 4). This level of 2,4-D is the level which is in current Institute use for initiation of loblolly pine. The curves seem to indicate that the charcoal becomes loaded with the maximum amount of 2,4-D in less than 24 hours, to ~170-180 ppm at 0.0625% AC and ~110-120 ppm at 0.125% AC. At 0.25% AC 12-17 ppm 2,4-D appear to be available at equilibrium. The important information to be drawn from this series is that the equilibrium value reached is ~ 13 ppm which is significantly higher than previously utilized concentration levels of 2,4-D found in the literature where successful initiation was recorded (9b,12,41). By examining the results obtained in the long-term series, it is evident that the majority of sorption occurs prior to the first sampling period. In an effort to better understand the adsorption phenomena in this system, an experimental plan focusing on the initial stages of adsorption was enacted next.

Less time will be spent in the analysis of the short-term series, as the primary goal was to observe if there were significant changes in the concentration of 2,4-D in the initial 24 hour period. In every case this is true with few exceptions. The exceptions occur when either the AC concentration is relative to the 2,4-D concentration or if the 2,4-D concentration overwhelms the charcoal. Outside of these two exceptions, the trends exhibited by the short-term series are similar to those found in the long-term series (Figures 5,6,7).

What was gained from the data is two-fold. It indicates that the current system (0.25% AC and 220 ppm 2,4-D) reaches an equilibrium at 12-17 ppm, whereas the 0.25% AC and 110 ppm 2,4-D stabilizes at 3-5 ppm. The current system exceeds the reported levels at which initiation occurred on clear media, and would seem to indicate that it would be beneficial to lower the initial 2,4-D concentration to 110 ppm. It is also apparent that the adsorption process is indeed very quick. The majority of the adsorption most likely occurs as the gel is still in it's hot aqueous phase while mixing, waiting to be poured (33,39). An experimental method needs to be developed which can focus on the critical first 15-30 minutes.

It is also noted that these two series show differences in the rates of adsorption. What was anticipated was that both series would overlap since the media components were at the same concentration levels and procedures and methods remained constant. In all of the graphs, the sorption line of the short-term series mimics the shape of the long-term series, but is shifted lower. At this time there is no reasonable explanation for the discrepancy between the two series. Several hypotheses concerning this deviation are discussed in the original A-190 document. One of the most plausible hypothesis concerns temperature. The first series was conducted during the winter and the second series during late spring. Room temperatures were lower in the first series than during the second series. Temperature may play a role in slowing or speeding adsorption.

As part of the project objectives, a developed practical research tool was desired. With the results that were collected, it is possible to begin to develop a rough descriptor of the conditions that were examined. In Table 2, the concentrations of 2,4-D and AC are given, as well as a range of available 2,4-D at several time intervals. The lower values come from the results of the short-term series, and the upper values from the results of the long-term series. As the short-term series was not examined beyond seven days, the data point at seven days will be treated as if it were an equilibrium value, although there is enough evidence to indicate that in some instances the system is not at equilibrium, and would still decrease. This does, however, give a frame of reference when comparing these results.

When directly comparing these results with the 2,4-D charcoal adsorption studies of Ebert and Taylor (10,11) one notices that their levels of available 2,4-D are significantly greater than the results in Table 2. This may be an artifact of their analysis procedure. In their study, they scraped 200 mg samples of medium from the surface. These scraping probably contained some charcoal particles which was subsequently introduced into the scintillation vial and placed in the counter. Some adsorbed 2,4-D likely contributed to their total estimations of available 2,4-D and may account for the differences in studies.

From Table 2, several interesting correlations can be developed which are an important first step in developing an empirical model to predict available 2,4-D levels based on initial 2,4-D concentrations, AC concentrations, and time. When the log of the concentration of available 2,4-D is plotted against AC concentration, coherent relationships within a time period become apparent (Figures 8-11). These linearized relationships exhibit strong correlation coefficients, ranging from 0.909-1.000. Three points in a line do not make a definitive solution, but are a good indicator of potential trends, and would provide validity in pursuing this problem further to develop a more rigorous solution. More data points are needed to legitimately portray any ensuing trends. Available 2,4-D is strongly correlated to the initial 2,4-D concentration levels at a specified charcoal level. The fact that correlations can be made between several of the variables involved in the process is a positive indicator that a final model may be developed which can encompass all of the designated variables: initial 2.4-D concentration, % AC, and time.

Conclusions and Recommendations

Although seemingly simplest in nature, the problem presented by the adsorption of 2,4-D by activated carbon in plant tissue culture media is a complex issue. To effectively develop an explicit mathematical model encompassing several potential variations within the time frame of the research period is ambitious at best. What can be concluded along these lines is that AC has an enormous adsorption capacity for 2,4-D within this media system. The initial concentration levels the Institute currently employs result in available 2,4-D concentrations that fall above the range where initiation has been reported in media not containing AC.

Further work is needed before these results are presented to the tissue culture community at large. While manipulating the initial concentrations of AC and 2,4-D to fall within the final 2,4-D ranges developed by this project is acceptable for the Institutes work in loblolly pine initiation media, extrapolating this information to a broader gamut of initiation mediums at this point is premature, yet obtainable.

Before any further work is invested along the lines of this project, a fundamental question must be answered as to where the destination of any resulting information may be. If the desired outcome is an intricate understanding of the behavior of AC in a plant tissue culture environment, there exists a vast amount of work in developing experimental procedures and analytical processes to answer many questions, of which only a few have been touched upon by this project. If the desired outcome is to develop a practical tool utilized by the Institute and tissue culture community, then procedures primarily developed within this research project can be used effectively to a great extent. It is completely plausible to develop a mathematical model based on empirical correlations of measured available hormone concentrations that will be able to predict available hormone levels based on initial concentrations, AC concentrations, and time, with some degree of accuracy.
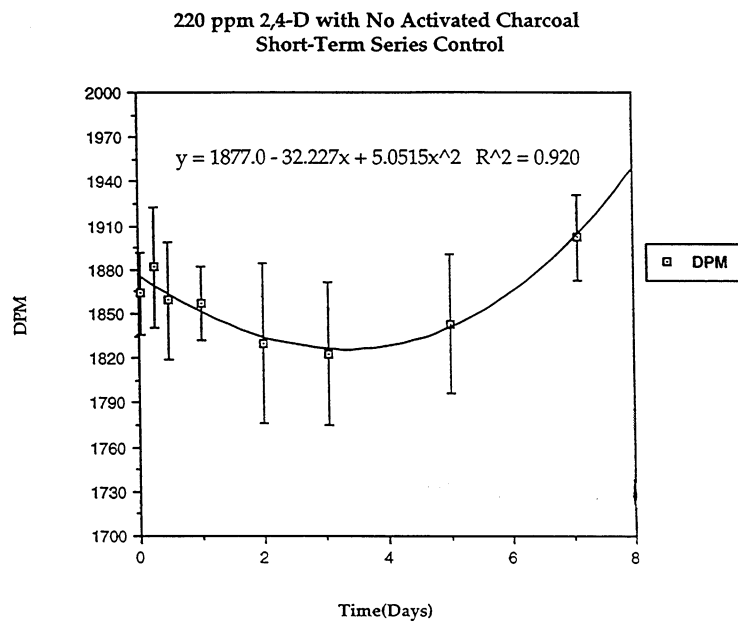
With respect to actual influence on initiation, and in light of this study, it is recommended that the initial 2,4-D concentration be dropped to 110 ppm at 0.25% AC. This level of initial 2,4-D yields stabilized concentrations values in the desired range, as implied by the literature.

Table i . Institute Initiation Medium 201 for use with Loblolly Pine(*Pinus taeda*).

	Medium Amt.
Macronutrients	**mg/L**
NH4NO3	200
KNO3	909.9
Ca(NO3)2.4H2O	236.2
CaCl2.2H20	0
KH2PO4	136.1
MgSO4.7H2O	246.5
Mg(NO3)2.6H2O	256.5
MgCl2.6H20	101.7
KCl	0
Micronutrients	
MnSO4.H20	10.5
ZnSO4.7H2O	14.4
CuSO4.5H2O	0.125
FeSO4.7H2O	13.9
Na2EDTA	18.65
H3BO3	15.5
NaMoO4.2H2O	0.125
CoCl2.6H2O	0.125
NiCl2.6H2O	0
KI	4.15
Carbohydrates	
myo-inositol	1000
Sucrose	0
Maltose	15000
Vitamins and AA	
Thiamine.HCl	1
Nicotinic Acid	0.5
Pyridoxine HCl	0.5
Glycine	2
L-Glutamine	450
Caseamino Acids	500
Hormones	
2,4-D	220
BAP	90
Kinetin	86
Additives	
Activated Charcoal	2500
Gelrite	4000

Table 2. Available 2,4-D concentrations(ppm) at three selected time intervals.

Initial 2,4-D(ppm)	AC (%)	1 Day(ppm)	7 Days(ppm)	14 Days(ppm)
110	0.0625	48.8 - 85.4	46.0 - 83.1	46.0 - 71.1
110	0.125	13.8 - 34.4	7.8 - 24.7	7.8 - 21.8
110	0.25	4.0 - 6.1	2.4 - 3.2	2.4 - 3.0
220	0.0625	141.0 - 173.3	138.9 - 200.0	138.9 - 188.6
220	0.125	59.7 - 112.4	49.2 - 126.0	49.2 - 117.3
220	0.25	11.8 - 20.8	7.5 - 13.7	7.5 - 14.6
440	0.0625	352.2 - 469.7	367.6 - 507.9	367.6 - 489.2
440	0.125	240.2- NA	225.9 - NA	225.9 - NA
440	0.25	72.4 - 198.2	65.1 - 182.0	65.1 - 160.5

220 ppm 2,4-D with No Activated Charcoal
Tail-end Series Control-Adjusted
Long-term Series

$$y = 1738.0 + 11.026x + 0.17524x^2 \quad R^2 = 0.933$$

DPM vs Time(Days)

DPM

220 ppm 2,4-D with No Activated Charcoal
Short-Term Series Control

$$y = 1877.0 - 32.227x + 5.0515x^2 \quad R^2 = 0.920$$

DPM

Time(Days)
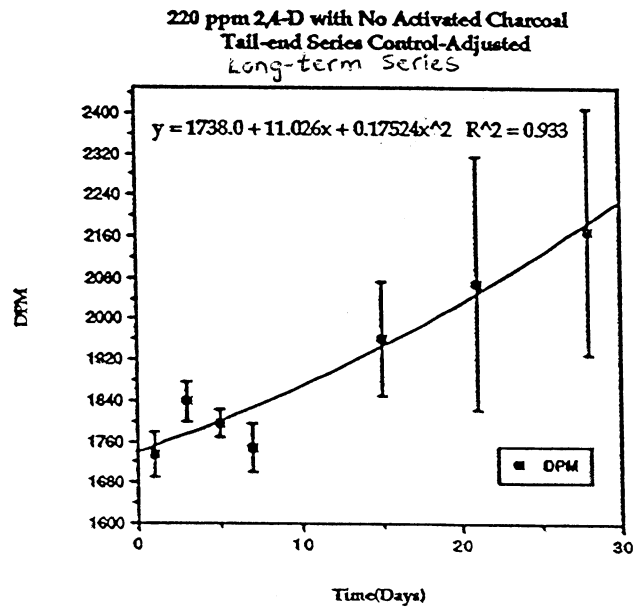
Figure 1. Actual data for long and short-term control media containing 220 ppm 2,4-D and no activated charcoa. A. Long-term control series. B. Short-term control series.
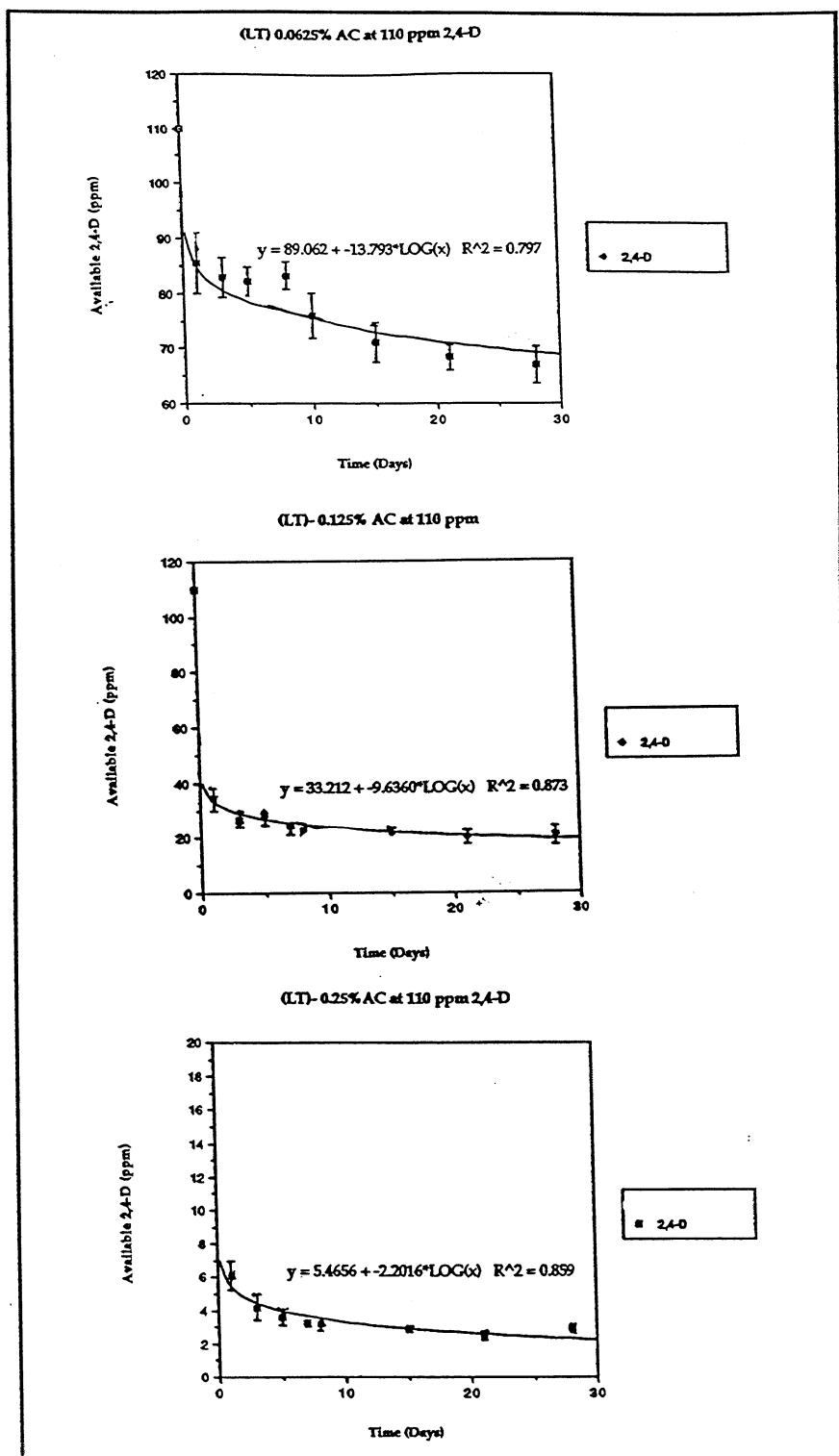
Figure 2 . Concentration levels of available 2,4-D observed at an initial concentration of 110ppm at three AC concentration levels(LT).

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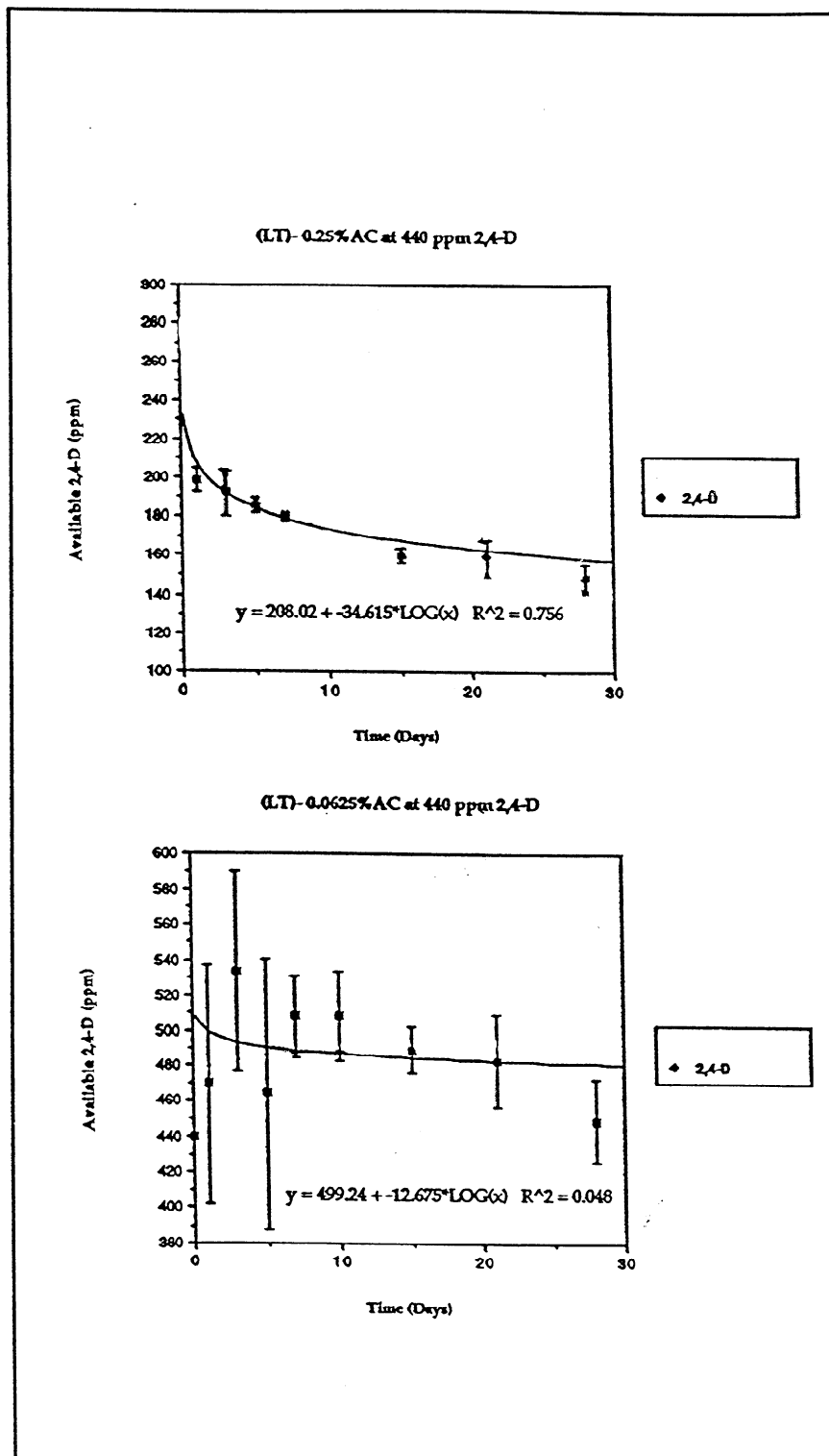
Figure 3 . Concentration levels of available 2,4-D observed at an initial concentration of 440ppm at two AC concentration levels(LT).

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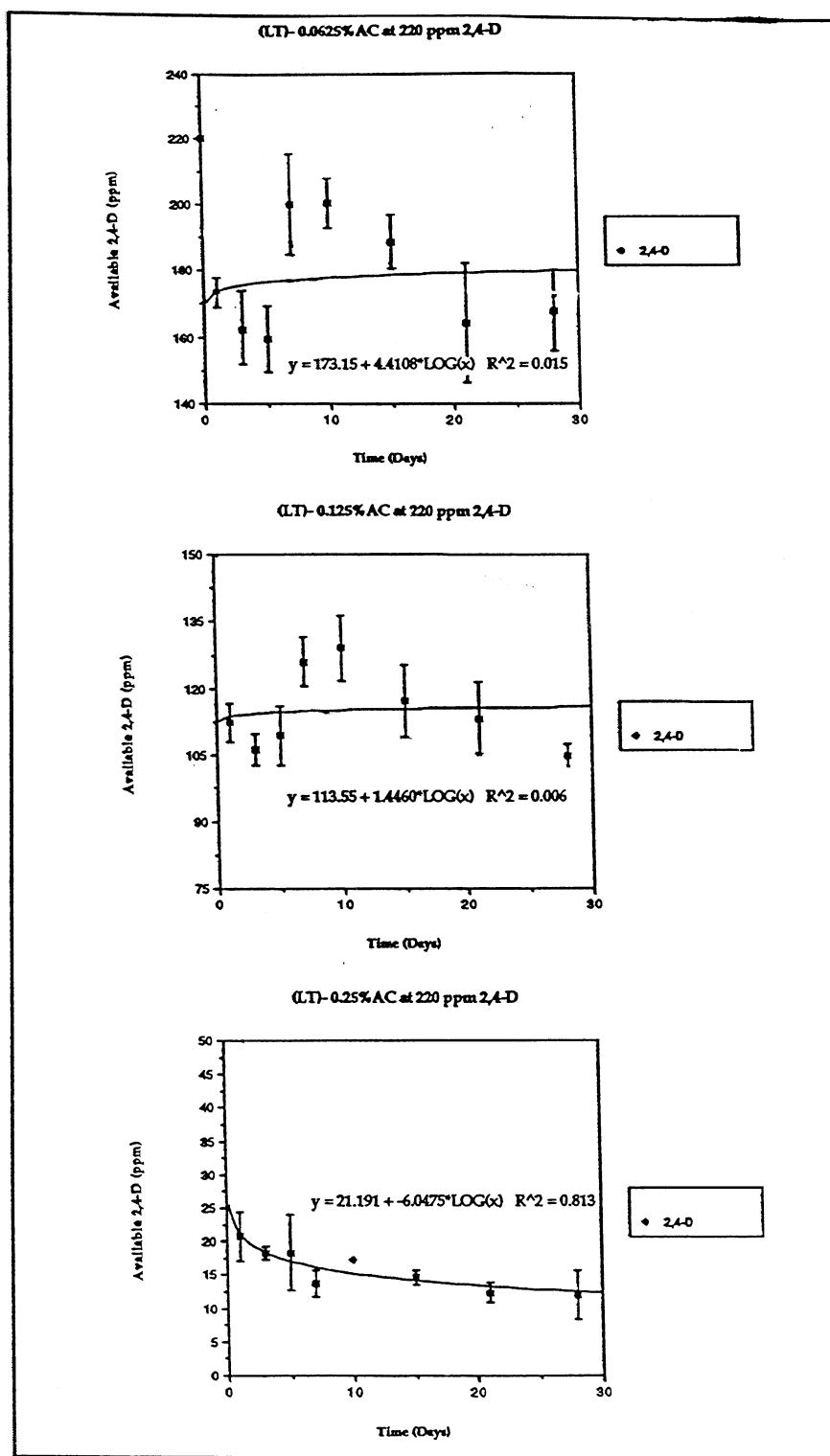
Figure 4. Concentration levels of available 2,4-D observed at an initial concentration of 220ppm at three AC concentration levels(LT).
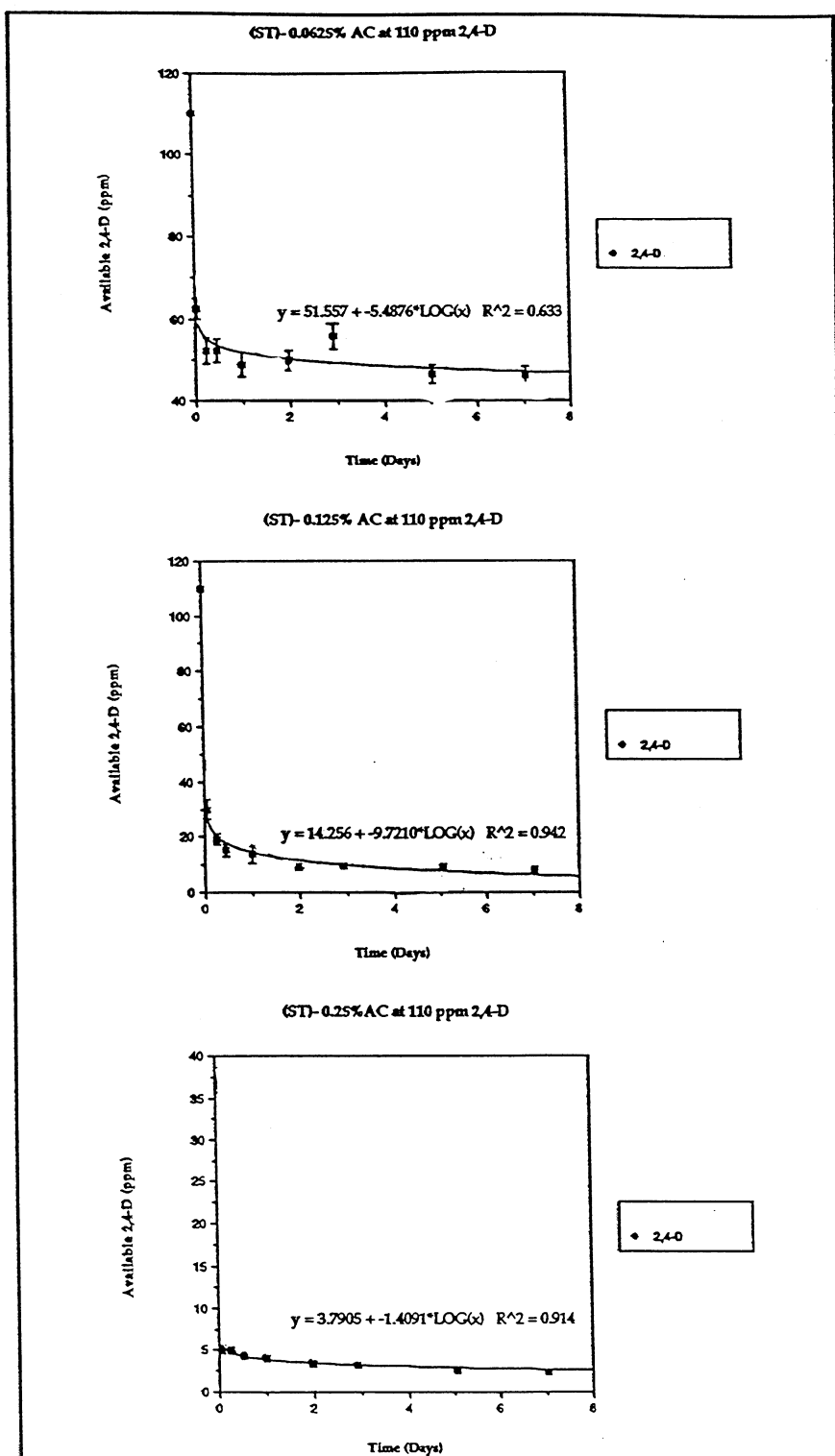
Figure 5. Concentration levels of available 2,4-D observed at an initial concentration of 110ppm at three AC concentration levels(ST).
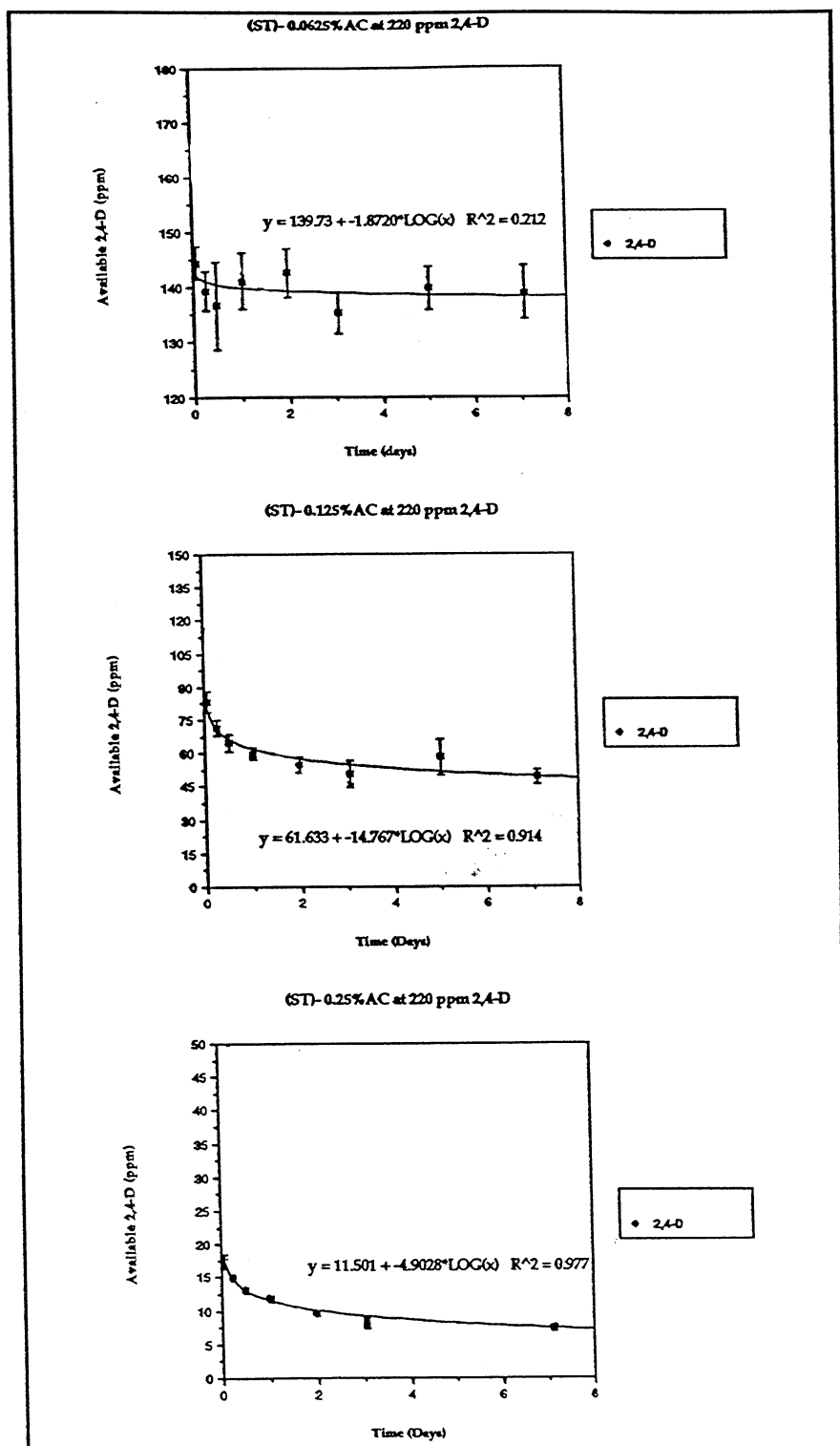
Figure 6. Concentration levels of available 2,4-D observed at an initial concentration of 220ppm at three AC concentration levels(ST).
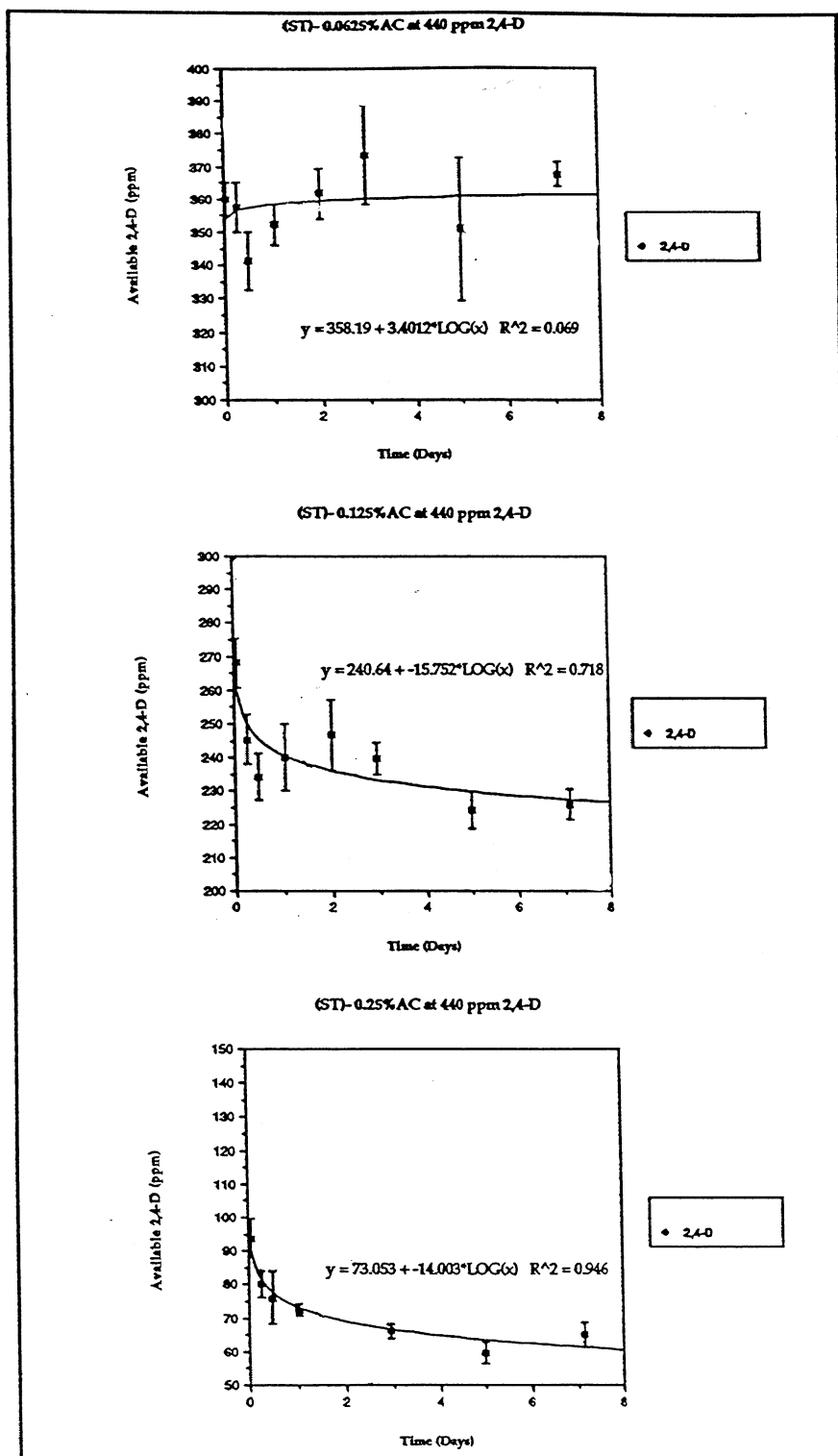
Figure 7 . Concentration levels of available 2,4-D observed at an initial concentration of 440ppm at three AC concentration levels(ST).
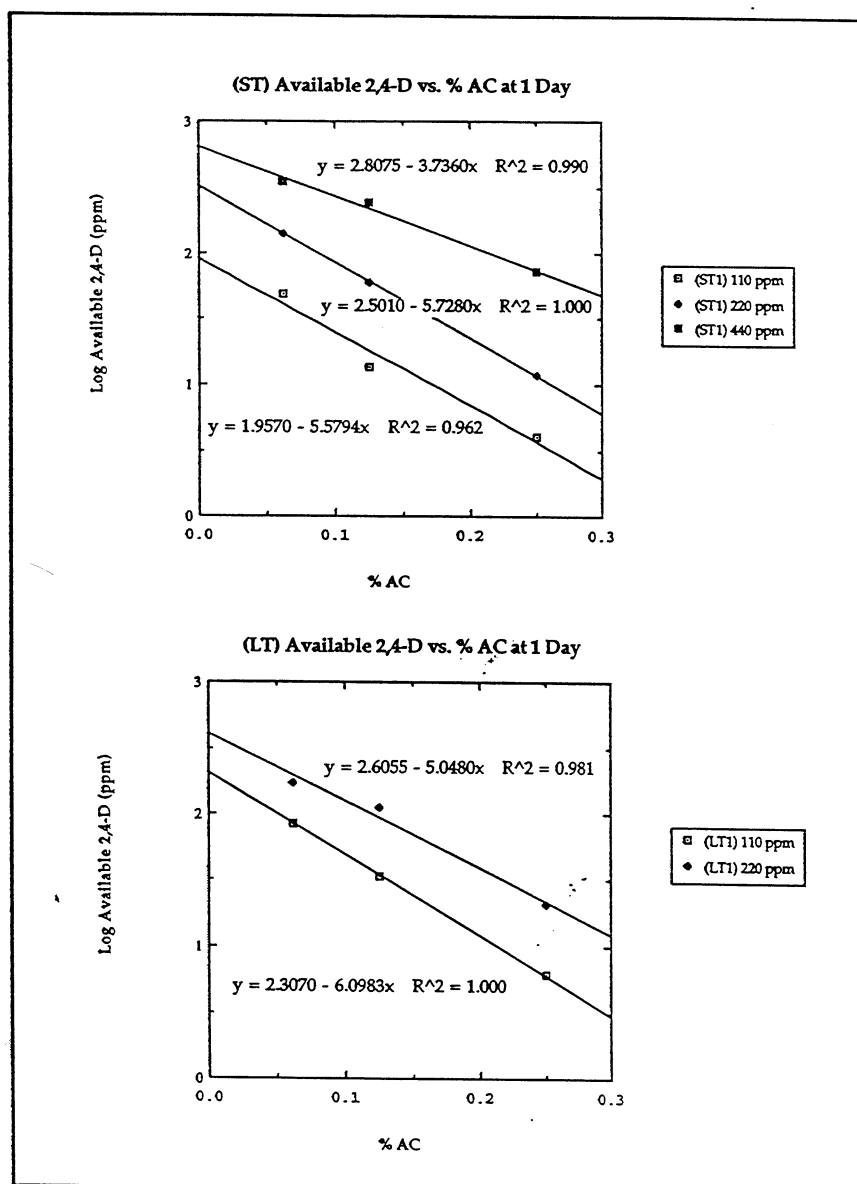
Figure 8 . Log available 2,4-D vs. % AC in the media at one day

(ST) Available 2,4-D vs. % AC at 7 Days

$y = 2.8350 - 4.0503x \quad R\char`\^2 = 0.997$

$y = 2.5515 - 6.7303x \quad R\char`\^2 = 0.999$

$y = 1.9190 - 6.4503x \quad R\char`\^2 = 0.909$

- □ (ST7) 110 ppm
- ◆ (ST7) 220 ppm
- ■ (ST7) 440 ppm

(LT) Available 2,4-D vs. % AC at 7 Days

$y = 2.7825 - 6.4217x \quad R\char`\^2 = 0.971$

$y = 2.3640 - 7.4834x \quad R\char`\^2 = 0.998$

- □ (LT7) 110 ppm
- ◆ (LT7) 220 ppm
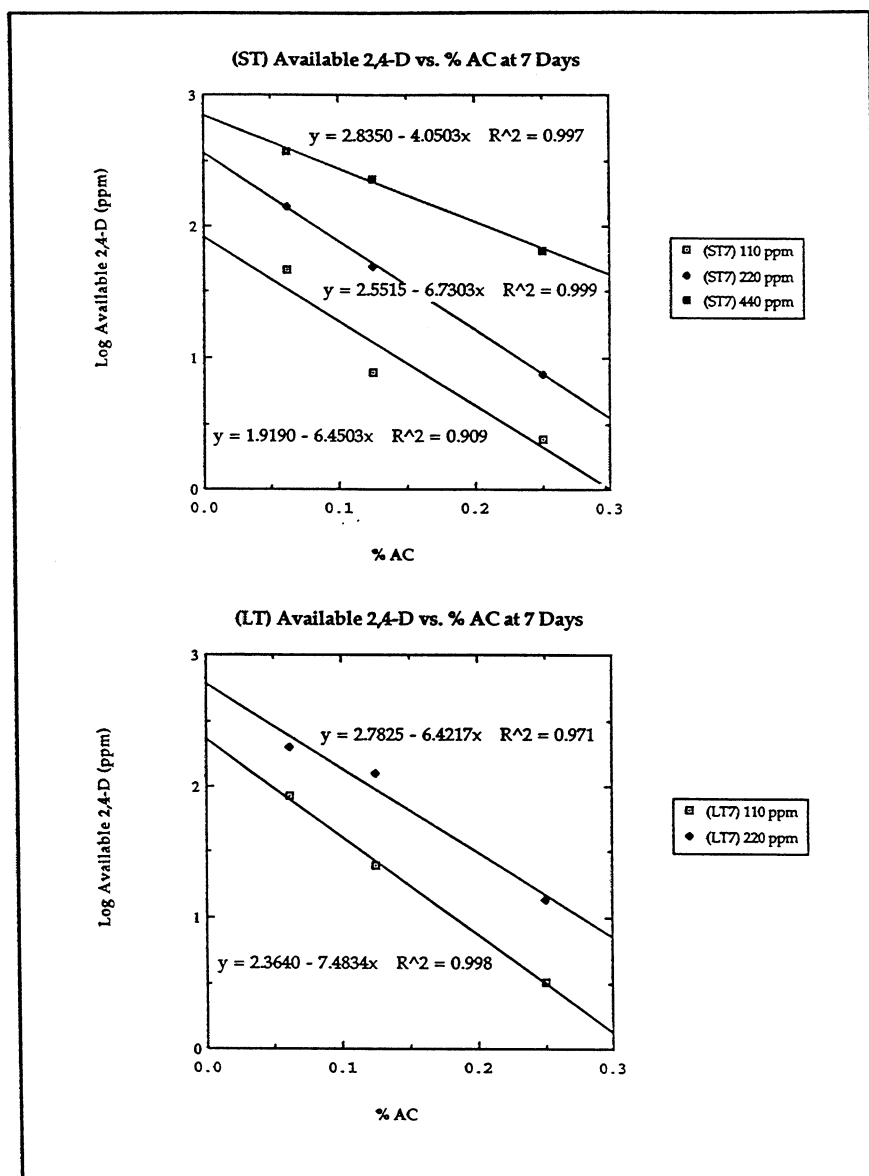
Figure 9 . Log available 2,4-D vs. AC % in the media at seven days

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(LT) Available 2,4-D vs. % AC at 15 Days

$y = 2.7285 - 6.1177x \quad R^2 = 0.975$

$y = 2.2825 - 7.2697x \quad R^2 = 0.998$

Legend:
- ☐ (LT15)110 ppm
- ● (LT15)220 ppm

Y-axis: Log Available 2,4-D (ppm)

X-axis: % AC

Figure 10. Log available 2,4-D vs. % AC in the media at 15 days

Figure II . Log available 2,4-D vs. initial 2,4-D concentration at 0.25% AC

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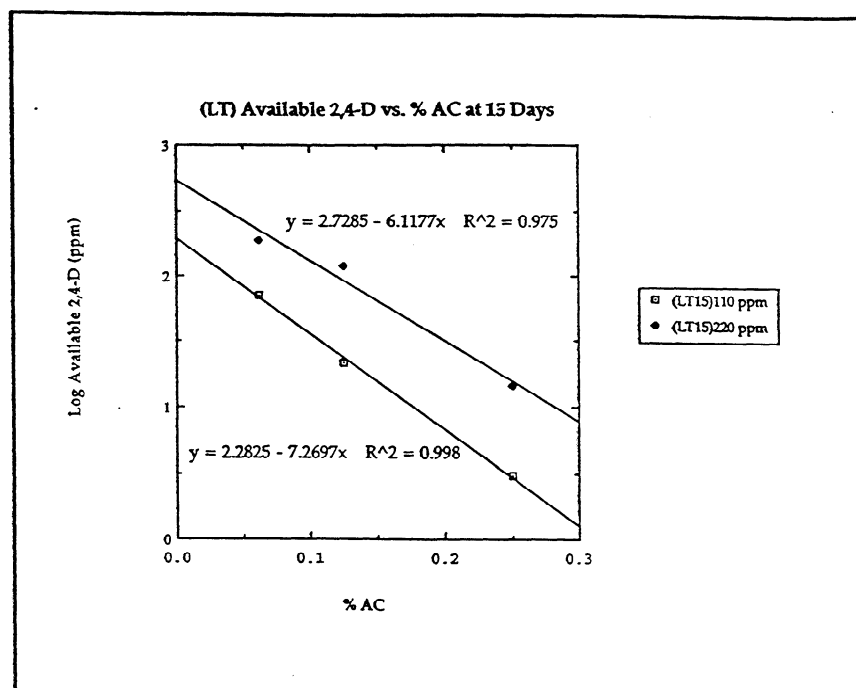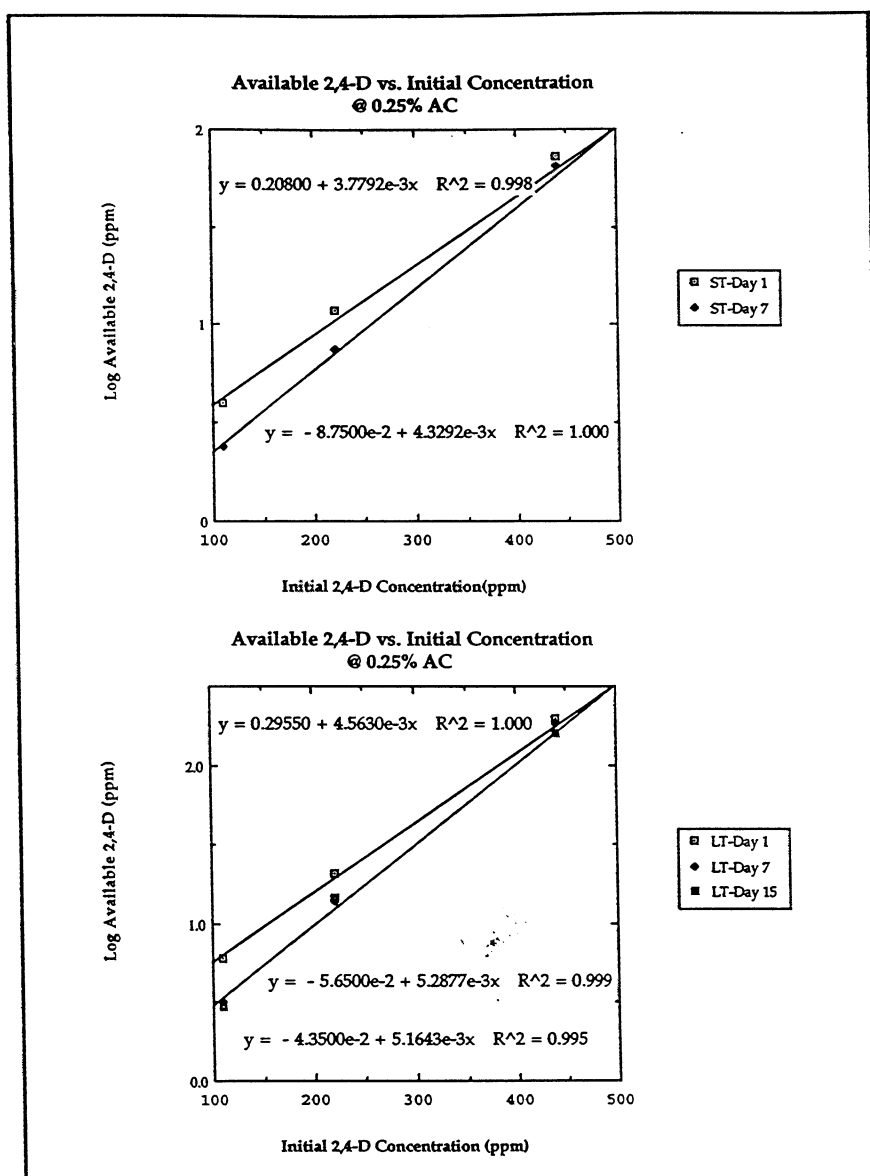
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MASS CLONAL PROPAGATION OF IMPROVED CONIFERS:

New Initiations - Culture

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS:
NEW INITIATIONS - CULTURE

Jerry Pullman
Shannon Johnson
Yolanda Powell

Summary

During the 1995 Summer approximately 440 cultures were initiated. Since we reported in previous PAC Meetings that loblolly pine cultures often loose their ability to form cotyledonary embryos over time, these new cultures provided an opportunity to determine how well our protocols work.

Twenty-five cultures which contained enough embryogenic tissue for starting liquid cultures were chosen for testing in one of our liquid maintenance protocols (medium 16) and one of our development and maturation protocols (medium 240). Liquid cultures were started as reported previously. Settled cell volumes and embryo stage ratings were taken weekly (Table 1). After five or more subcultures, settled cells were plated with one ml per plate on development medium 240. Plates were observed for embryo development and evaluated for the latest stage visible. Embryos were still developing during the last observation shown in Table 1.

Data in Table 1 show that out of 25 cultures tested 18 or 72% were able to start liquid cultures. Two of the seven cultures that did not start were re-tested for the ability to start a liquid culture and were successful bringing the overall liquid culture success rate to 80%. The remaining five cultures had stopped growing in the maintenance medium and were no longer available. Average weekly multiplication rates over five weeks ranged from 1.5 to 4.7 times increase per week. Average weekly embryo stage ratings varied from 1-2.9 (see embryo classification system reported in the March 21, 1994 PAC Report). Maximum stage ratings of at least 2 were seen in 16/25 cultures (64%) and maximum early-stage embryos at stage 3 were seen in 5/25 cultures (25%). The maximum stage rating is important because previous observations showed that liquid cultures with stage ratings of two or higher had the best chance of producing cotyledonary embryos. At the time this section was written cotyledonary embryos had developed and were still developing in at least 6/15 (40%) of the cultures tested. Table 1 shows the embryo stage visible, approximately how many embryos had formed, and time period on development and maturation medium 240.

Table 2 shows an overall summary of loblolly pine somatic embryogenesis at IPST. Major progress has been made in the areas of initiation, maintenance, embryo development, germination, and conversion. However, there are many challenges ahead particularly in the area of embryo quality. Embryo quality and vigor must be improved during all stages for the somatic embryogenesis process to become commercial. With the successful storage of cultures in liquid nitrogen shortly after initiation we hope to prevent any culture decline over time, decrease the labor to maintain cultures, and have available a bank of cultures with known histories of performance in the somatic embryogenesis process. Research could then focus on the particular step or embryo stage where improvement is needed.

Table 1. Loblolly pine culture performance: starting liquid cultures, weekly growth rate as settled cell volumes, embryo stage in liquid culture (medium 16), and maximum stage rating in development and maturation medium (medium 240).

Culture #	Origin	Weekly Settled Cell Volume (5 wk avg.)	Liquid Stage Rating - (5wk avg/maximum rating	Development Media - Most Advanced Embryo Stages (Yield/ ml Cells)	Weeks on Development Medium when evaluated
245	UC5-1036	2.4	1.7 / 2	6, very small	6 weeks
246	UC5-1036	1.6	1.4 / 2	4	3 weeks
247	UC5-1036	1.8	1.7 / 2	8 (1-5/ml)	6 weeks
248	BC-3	No Start[2]			
249	WV-F2	3.2	2.0 / 2.5	8 (1-5/ml)	6 weeks
250	WV-F2	3.6	1.7 / 2	2	6 weeks
251	WV-F2	No Start[1]			
252	BC-9	2.7	2.5 / 3	6-7	4.5 weeks
253	UC10-33	4.6	1.7 / 2	3	6 weeks
254	UC10-33	2.0	1.8 / 2	3	4.5 weeks
255	BC-9	1.8	2 / 2	8 (10-30/ml)	4 weeks
256	WV-F2	1.3	1.8 / 2	not tested yet	
257	UC10-33	3.2	2.1 / 3	6-7 (10-30/ml)	6 weeks
258	UC10-5	No Start[2]			
259	BC-9	2.1	2.9 / 3	8 (10-30/ml)	4 weeks
260	BC-3	1.5	2 / 2	not tested yet	
261	UC5-1036	1.4	2.2 / 3	8 (10-30/ml)	4 weeks
262	WV-I2	1.9	1 / 1	not tested yet	
263	UC10-5	No Start[2]			
264	WV-F2	No Start[2]			
265	UC5-1036	No Start[1]			
266	UC10-33	2.3	2.7 / 3	7-8 (10-30/ml)	6 weeks
267	UC10-33	2.4	1.6 / 2	5-7 (10-30/ml)	6 weeks
268	UC5-1036	4.7	1.5 / 1.5	6 (1-5/ ml)	6 weeks
269	BC-2	No Start[2]			
Average		72% 1st Start 80% 1st & 2nd Start	2.5 / 2.2	40% cultures have stage 8 embryos	

[1]/ Upon a second try liquid cultures were successfully started.
2/ Tissue was unavailable for a second attempt to start liquid cultures.

Table 2. Summary of loblolly pine somatic embryogenesis at IPST, past performance (1992), present performance (1996) and future goals for the major steps in the process.

Somatic Embryogenesis Step	Where we were 1992	Where we are now 1996	Goal
Initiation			
Overall		6.5%	
Best Media (505)	<3%	16.5%	35%
Maintenance			
On Media			
Survival	?	20%	50% of initiated
Growth	Gelled 2x / month	Liquid (2.5x / wk)	OK
Embryo quality	?	Stage 2-3 (64%)	OK
		Loss over time	Stop loss in Liq N$_2$
Cryogenic Storage	Not tested	Equipment recently available. Tests to begin shortly	80% Survival
Development			
Yield (stg 6+)	1-5 embryos / ml	10-30 embryos/ml	OK
Quality	stage 7-8	stage 8+	stage 9.4
Genotypes	2-3 genotypes produced stage 7-8	6/15 (40%) produce stage 8	50% liquid cultures
Germination (shoot & root growth)			
Percent	None	5-50%	50% liquid cultures
Genotypes	None	2 genotypes (195, 230)	50% liquid cultures
Conversion			
Percent	None	94% (genotype 195)	80% of germinants
Genotypes	None	1 genotype (195)	50% liquid cultures

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS:

Long-Term Storage of Loblolly Pine Somatic Embryo Cultures in Liquid Nitrogen

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS: LONG-TERM STORAGE OF LOBLOLLY PINE SOMATIC EMBRYO CULTURES IN LIQUID NITROGEN

Jerry Pullman
Shannon Johnson
Yolanda Powell

The Forest Biology Group recently obtained a state-of-the-art cryogenic storage system. This equipment will enable the somatic embryogenesis program to economically store initiated cultures for long periods without culture change or loss of embryogenic capacity. In addition, the significant labor costs to repeatedly maintain large numbers of cultures weekly, bimonthly, or even monthly will be largely decreased.

Cryogenic storage equipment is used to maintain long-term cell viability at ultra-low temperatures. Water is the major component of all living cells and must be present in order for chemical reactions to occur within a cell. During cryopreservation, the water changes to ice and cellular metabolism ceases. Dehydration also occurs changing the concentration of salts and other metabolites, causing an osmotic imbalance which can be detrimental to cell recovery. These detrimental effects can be minimized by controlling the rate of cooling, using cryo-protective agents, and maintaining appropriate storage temperatures and rates of re-warming. The purchased cryogenic storage equipment causes the slow decrease of temperatures suitable for submersion in liquid nitrogen, provides storage space in liquid nitrogen and provides safe handling and temperature monitoring equipment. Liquid nitrogen facilities are available at local medical and engineering research facilities and at Atlanta Sperm Banks. However these facilities are oriented towards human and animal cell storage. Supporting equipment at these local facilities, such as sterile air-flow hoods, were either not available or available with difficult accessibility. There are no cryogenic storage facilities dedicated to plant research in the North Georgia area.

An added benefit to cryogenic storage of loblolly pine early-stage embryo cultures may be the selection of embryogenic tissue. Reports of angiosperms and Douglas-fir [Gupta et al., 1994. Plantlet regeneration via somatic embryogenesis in Douglas-fir (*Pseudotsuga menziesii*). TAPPI R&D Division Biological Sciences Symposium, October 3-6, 1994, Minneapolis, Minnesota, pp. 35-39.] show improved subsequent embryo development after cryogenic storage. The current hypothesis is that cryogenic storage eliminates competing non-embryogenic tissue within a culture. It is likely that the same mechanisms will improve loblolly pine embryogenic cultures.

CryoMed cryogenic storage equipment was chosen due to reports of high rates of successful natural and somatic embryo storage in liquid nitrogen {P. M. A. Toivonen and

K. K. Kartha. 1989. Cryopreservation of cotyledons of non-germinated white spruce [*Picea glauca* (Moench) Voss] embryos and subsequent plant regeneration. Journal of Plant Physiology, Volume 134, pages 766-768.}

Description of the research Instrumentation (Shown in Figure 1.)

Equipment by CryoMed (by Forma Scientific, Inc.)

a. #1050 Computer Interface Freezing System
Includes: Freezing rate controller, freezing chamber, complete monitoring equipment, computer programs for monitoring, and hose for liquid nitrogen connections.

b. #8175 CryoPlusII Liquid Nitrogen Storage Unit
Contains space for 10,000 2.0 ml storage vials, has necessary nitrogen level and temperature monitoring equipment.

c. essential storage racks and vials, Filling Racks, and appropriates safety equipment:

Figure 1. Cryogenic storage equipment. From left to right: Liquid Nitrogen Storage Unit, Liquid Nitrogen Tank, Freezing Chamber, Computer (top), Printer (middle), Freeze Rate Controller (bottom).

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS:

Conversion of Loblolly Pine Somatic Embryo Germinants to Established Plants

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

Jerry Pullman
Barbara Johns
Shannon Johnson
Yolanda Powell

The Forest Biology Group previously reported the conversion of Loblolly Pine somatic germinants to established plants growing under high humidity conditions. Since the Spring of 1995 94% of the plants exposed to ambient greenhouse conditions have survived. Presently 61 converted plants of genotype 195 are maintained in an open greenhouse. Of the many factors which affect conversion two have emerged as the most influential: germinant quality and planting in a closed growing system.

The highest percentage of established plants has been found to come from vigorous germinants with an epicotyl and root of at least 2.0 mm in length. Less developed germinants rarely survive. To date the closed system is still the best method for growing somatic plants. The protocol has been modified and we are currently experimenting with planting germinants directly into leach tubes in plastic racks. A metal frame inserted into both ends of the rack is covered with a plastic bag. The closed system is placed under un-screened lamps in the light culture room. The germinants are regularly fertilized with 1/4 strength Schenk and Hildebrant basal salts.

Germinants of a new genotype, 230, which exhibited shoot and root growth were planted in magenta boxes for evaluation of conversion. Table 1 shows an update of somatic seedlings of loblolly pine which are in various stages of conversion.

Table 1. Somatic Seedlings of Loblolly Pine Under Conversion

Somatic Seedlings of Loblolly Pine Under Conversion - Type of Environment, Genotype, and 1/2 Sib Family	Number of Plants
Greenhouse (open Environment)	
Genotype 195 (UC10-1003)	61 plants
Somatic Seedlings in Soil but acclimating to open Environment	
Genotype 195 (UC10-1003)	11 plants
Somatic Seedlings - New Conversions	
Genotype 195 (UC10-1003)	45 plants
Genotype 230 (UC10-14)	29 plants

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

(Molecular Biology)

John Cairney
Shujun Chang
Karen Floyd
Bobbie Johns
Jeff Grass
Brian Klunk
Tom Kraker
Debbie Villalon
Byron Waldrop

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS -
MOLECULAR BIOLOGY PROJECT UPDATES

<u>Gene Expression and Somatic Embryogenesis - Pine.</u>

In previous PAC presentations the advantages of Differential Display as a tool to monitor somatic embryo development have been discussed. Our most recent research goals have been;

1. To establish the technique of Differential Display with Pine tissue
2. To apply that technique to Pine Somatic Embryos

1. To establish the technique of Differential Display with Pine tissue

Our initial attempts used large (gram) amounts of pine tissue. In this case we isolated RNA from the needles, stems and roots of the same 8-month old pine seedling. Our normal RNA isolation procedure was employed. This RNA was treated with DNase, to remove contaminating chromosomal DNA, phenol/chloroform extracted and was reversed-transcribed using MMLV-Reverse Transcriptase. The primer for synthesis was a modified oligo dT , MAT12 . This primer is designed to hybridized to the beginning of the polyA tail of transcripts where the ribonucleotides adjacent to the tail are N (any) and T. A subset of the mRNA is reverse-transcribed in this reaction. One tenth of the reaction volume was used for PCR. A random 12-mer primer, designated AP1, and an additional aliquot of MAT12 were used in a reaction volume of 20mL. Five mL of this reaction was separated on an 8% polyacrylamide 'sequencing gel'. The gel was dried and exposed to film. After 36 hours exposure strong signals appeared. Needle-, Stem-, and Root-specific bands could be discerned as well as bands which showed equal intensities and unequal intensities for each tissue. This result indicated that we were successful in discriminating patterns of gene expression in pine tissue using Differential Display.

2. To apply that technique to Pine Somatic Embryos

We now wished to apply the technique to Pine Somatic embryos. Our first efforts using somatic embryos for Stage 1 and Stage2, used a commercial RNA preparation method which had been designed for use with small amounts of tissue. This was used since we feared that we would lose most of our RNA during precipitation if our usual RNA isolation method was used. Using the same RT and PCR methods as described above a very few, very feint bands were visible. Protocols were modified but results obtained were not significantly better. We returned to our usual RNA isolation method, using One gram of Somatic Embryos (Stage 6-7, stage 2 and stage 4-5). This method, combined with protocol modifications gave many strong bands and bands unique to particular stages could be observed.

Our current goals are;

1. to establish the reproducibility of our methods,
2. to assay, systematically, gene expression in the embryos we have in hand
3. to attempt these assays with smaller amounts of tissue (with the ultimate goal of assaying gene expression in a single embryo)

Project Update:

We have conducted DD with early stage somatic embryos of different genotypes and are maintaining these genotypes. At present we are using early stage embryos and isolating RNA from 0.6 - 1 g of embryos (corresponding to ca. 10ml settled cells). We have used an RNA isolation method developed in this lab (Chang et al. 1993. Plant Mol. Biol. Reporter 11: 113-116). Yields have ranged from ca. 60-150ug RNA/g embryos. The variation is probably because the weight of isolated embryos is difficult to estimate, SEs do not compact well on settling and centrifugation can damage embryos, thus even when media removal and snap-freezing is done carefully, some media will be present in embryo preparations and will contribute to the "Weight".

Embryos at different stages of maturity are being harvested, RNA isolated and DD performed. Side by side comparison of different stages and different genotypes should reveal gene expression differences which will then be assayed further to determine their reproducibility. By comparing genotypes which give an abundance of high-quality embryos with those which do not advance beyond early stages we will identify major differences in gene expression and attempt to relate specific gene expression to competence to form embryos. We will isolate bands which are associated with "Good" embryo formation and characterize them by Northern analysis, cloning and sequencing.

Program Building:
Several people have joined our group recently to work on the Differential Display/Somatic embryogenesis project. This aspect of the Softwood genetics program will now become a major thrust of the molecular biology at IPST. The new project members are:
Vincent Ciavatta - Ph.D. Student
Byron Waldrop - MS Student.
New Post-Doc - to be hired (just got money)
Bobbie Johns - Senior Tech, plating and maintaining SEs for DD

Drought-stress protection/factors affecting gene expression

Pine - We are conducting gene expression assays using both seedlings and tissue cultured early stage somatic embryos. Eight-month old seedlings which had been subjected to wounding, heat, cold-, and drought-stress. We have also exposed cells in tissue culture to

ABA and Jasmonic Acid, two plant growth regulators which are involved in stress-related gene expression in many cases. RNA has been isolated from the needles, stems and roots of stressed seedlings and from cells growing in the presence of hormones for varying periods of time. Slot-blots and image scanning of membranes are being conducted to gain both quantitative and qualitative assays of gene expression.

Atriplex - As stated before, both the RNA and the encoded protein of the defense/repair gene which we are studying in Atriplex (Proteinase Inhibitor-I) have novel features. These features may be exploited to design broad range plant transformation vectors as well as produce plants of enhanced tolerance. Cell culture experiments with ABA and PEG have been conducted and slot blot assays are being analyzed.

Recently a collaboration has been established with Dr. Jim Powers at Georgia Tech. Dr. Powers is a world-renowned researcher who specializes in Proteinase Inhibitors and his laboratory will conduct activity assays using peptides of the active site of our Proteinase Inhibitor. From this work we can determine the kinetic parameters of the enzymes encoded which we possess. These clones are very similar but contain a number of differences the significance of which we will be able to assess. This information will allow us to choose the most active form of the enzyme for transformation. Further they will open the way for genetic engineering experiments wherein we can tailor and enzyme to act optimally under a given condition.

Project Update:

Hormone treatment of whole plants and plant cell culture was conducted and using RT-PCR and Northern analysis, induction of expression was shown for a number of the genes in the PI gene family. Sequence analysis reveals, in the genomic clones, a number of motifs which correspond to Transcription Factor binding sites, whose number, sequence variation and location could explain the difference in the magnitude of response to different elicitors.
Preliminary results of peptide assay, conducted in the laboratory of Dr. Jim Powers (Georgia Tech), show that the core peptide of the Proteinase Inhibitor 8-3 shows affinity for Proteinase. Affinity is low, however further experiments, using a cyclized version or the peptide are being tried. This preliminary result, however, does appear to demonstrate that the protein encoded by 8-3 is a proteinase inhibitor, confirming the prediction arising form homology with other proteins.

These investigations will be extended and expanded by conducting experiments in transgenic plants. Promoters and other elements which may control gene expression will be cloned into plant transformation vectors and transferred into plants. The potential for protecting Poplar by way of over expressing PI proteins suggest that this would be a likely host plant. Gene expression studies will initially be conducted in fast growing plants such as Arabidopsis or Tobacco and information gathered from these experiments can then be applied to tree transformation.

Manipulation of Lignin Synthesis

This project has three strands;

1. To transfer sense and antisense genes into plants to modify lignin synthesis.
2. To isolate a promoter of a Loblolly Pine O-Methyltransferase gene involved in Lignin synthesis.
3. To isolate stem specific promoters which could be used to drive expression of antisense genes

To determine whether the gene was being expressed, RT-PCR was conducted using RNA from two of the transformants. In each case a single strong band was observed however this was of an unexpected size. No such band was seen in a non-transformed control. As yet, we do not understand this result.

We intend using a novel PCR-based technique to generate the specific promoter for our gene of interest. We have designed an oligonucleotide based on the sequence of the a cDNA clone which we isolated previously. Linearized vector will be ligated to digested chromosomal DNA. A primer to the vector and the gene-specific primer will be used to amplify the OMT promoter from among the ligation products. This project is being conducted by MS student, Brian Klunk.

Project Update:

DNA has been isolated cut and ligated to vectors. PCR experiments are being conducted to amplify the fragments of interest. Meanwhile Southern analyses are being carried out to determine gene copy number for pLP1.

A second MS project, this time from student Tom Kraker, focuses on isolation of a stem-specific promoter. The rationale derives from concerns that a general reduction of lignin synthesis may compromise the plants defense capabilities. A stem specific promoter, not involved, specifically in the defense reaction, may be a more appropriate promoter for driving an antisense gene. Differential display experiments designed to identify stem-specific transcripts have been successful in establishing the technique though in the first few experiments few good candidate stem-specific mRNAs were identified. The experiments are being repeated using different oligonucleotide primers. Once identified, the bands will be isolated and sequenced to determine their identity.

Project Update:

This aspect of the project has moved apace. Using DD, strong, reproducible differences between gene expression in the stems, needles and roots of unstressed loblolly pine have been identified. This work has allowed us to survey patterns of gene expression with a view to identifying genes whose promoters may be used to express desirable proteins in transgenic plants. The original intention was to isolate cDNAs of genes expressed exclusively or preferentially in the stem. Several of these bands were identified and excised from the acrylamide (sequencing) gel and the DNA eluted from the gel by boiling. The quantities of DNA in solution at this point are small therefore a second round of PCR is conducted, using the same primer set, but this time the sole template is the cDNA eluted from the gel. If successful, products of this reaction should be in sufficient quantity to be viewed on an Ethidium Bromide-stained agarose gel. When independent experiments were conducted with six putative stem-specific bands eluted from the acrylamide gel, PCR produced a single strong DNA band in each case, easily visible on agarose gels. The size of these bands ranged from 150bp to 300bp. These bands were the only product of PCR and there was no background smear. These cDNA fragments will be cloned and characterized further to confirm the pattern of expression, through Northern analysis, and to determine their identity, through DNA sequencing. Additional bands were selected, which showed a root-specific expression or an even, high-level of expression in all tissues. These too will be analyzed.

The apparent success of this technically challenging extension of Differential Display is a major advance for the program as a whole. This technique will be applied in the Somatic Embryogenesis and Sterility Programs.

Genetic Engineering of Sterility in Southern Tree Species

Project Update:

Concerns about the dispersal of transgenes through pollen have led various groups to attempt to manipulate flowering by genetic means. A number of genes which affect flower development have been identified and many of these encode transcription factors. The conservation of sequence of these genes between species has allowed PCR amplification and cloning of homologous genes from tree species (Strauss et al 1995, Molecular Breeding 1:5-26). Most of this work has focused on species of interest to the Pacific Northwest. We have begun a project to clone important floral homeotic genes from Loblolly Pine and Cottonwood, commercially important species for the Southern US. We have designed oligonucleotide primers based on the sequence of LFY (Kelly et al. (1995), Plant Cell 7, 225-234) and AG (Rounsley et al. (1995), Plant Cell 7,1259-1269). These primers correspond to regions of the genes which are strongly conserved. If gene sequences are conserved we should be able to generate the homolog of LFY by PCR.

Preliminary PCR experiments, using LFY primer pairs and chromosomal DNA from loblolly pine show an amplified DNA fragment of just under 200 nucleotides, this is close to the size of the fragment, (180 nucleotide) which would be generated from Arabidopsis DNA. Background signal is high and these experiments are being repeated and optimized. Similarly a fragment of around 120 nucleotides can be seen using AG primers, again an expected size.

Table 1. Genes Involved in Floral Development in Arabidopsis and Antirrhinum

Arabidopsis Gene Locus	Antirrhinum Homolog	Mutant Phenotype	Proposed Molecular Functions(s)	Reference [a]
AGAMOUS (AG)	PLENA (PLE)	Homeotic conversion of stamens to petals and of carpels to sepals.	Putative transcription factor and negative regulator of AP1, AP2, and AP3.	1 2
APETALA1 (AP1)	SQUAMOSA (SQUA)	Homeotic conversion of sepals to leaves absence of petals.	Putative transcription factor.	3, 4, 15
APETALA2 (AP2)	ND (b)	Homeotic conversion of sepals to leaves or carpels and petals to stamens.	Negative regulator of AG.	5, 6
APETALA3 (AP3)	DEFICIENS (DEF)	Homeotic conversion of petals to sepals and of stamens to carpels.	Putative transcription factor and negative regulator of PI.	7, 8
PISTILLATA (PI)	GLOBOSA (GLO)	Similar to AP3.	Putative transcription factor and positive regulator of AP3.	9, 10
LEAFY (LFY)	FLORICAULA (FLO)	Partial conversion of floral meristems to inflorescence shoots.	Putative transcription factor and positive regulator of AP3 and PI.	11, 12, 13
CAULIFLOWER (CAL)	ND	Conversion floral meristems to inflorescence meristems.	ND	14

(a) (1) Yanofsky et al. (1990), Nature **346**, 35-39; (2) Bardley et al. (1993), Cell **72**, 85-95; (3) Mandel et al. (1992), Nature **360**, 273-277; (4) Huijser et al. (1992), EMBO **11**, 1239-1249; (5) Gustafson-Brown et al. (1994), Cell **76**, 131-143; (6) Weigel (1995), Plant Cell **7**, 388-389; (7) Jofuku et al. (1994), Plant Cell **6**, 1211-1225; (8) Jack et al. (1992), Cell **68**, 683-697; (9) Schwarz-Sommer et al (1992), EMBO J. **11**, 251-263; (10) Goto and Meyerowitz (1994), Genes and Development **8**, 1548-1560; (11) Trobner et al. (1992); (12) Weigel et al. (1992), Cell **69**, 843-859; (13) Huala and Sussex (1992), Plant Cell **4**, 901-913; (14) Coen et al. (1990), Plant Cell **5**, 1175-1181; (15) Kempin et al. (1995), Science **267**, 522-525.

(b) ND, not determined

Table 2. Genes Involved in Floral Development in Other Plant Species (Including Trees)

Plant	Gene Locus	Homolog	Proposed Molecular Function(s)	References (a)
Petunia	Floral Binding Protein1 (FBP1)	*PISTILLATA / GLOBOSA.*	Putative transcription factors for petals and stamens.	1
	Floral Binding Protein2 (FBP2)	*APETALA3 / DEFICIENS.*	Putative transcription factor for petals, stamens, and carpels.	1
	Floral Binding Protein7 (FBP7)	*AGAMOUS.*	Putative transcription factors for carpels.	2
	Floral Binding Protein11 (FBP11)	*AGAMOUS.*	Putative transcription factors for carpels.	2
Tomato	Tomato AGAMOUS1 (TAG1)	*AGAMOUS / PLENA.*	Putative transcription factor for stamens and carpels.	3
	Tomato MADS box gene no. 5 (TM5)	*APETALA3 / DEFICIENS.*	Putative transcription factor for petals, stamens, and carpels.	4
Tobacco	Nicotiana FLO/LFY (NFL)	*FLORICAULA / LEAFY*	Putative transcription factor for meristem identity.	5
Black Cottonwood (*Populus trichocarpa*)	*LFY*	*FLORICAULA / LEAFY*	Putative transcription factor for meristem identity.	6
Eucalyptus (*Eucalyptus globulus*)	*EUCLEAF 1, EUCLEAF 2, EUCLEAF 3*	*FLORICAULA / LEAFY*	Putative transcription factor for meristem identity.	7
Black Spruce (*Picea mariana*)	Numerous MADS-related genes	*AGAMOUS SQUAMOUS*		8
Norway Spruce (*Picea abies*)		*AGAMOUS-LIKE (AGL2)*		9

(a) (1) Angenent et al. (1992), Cell **4**, 983-993; (2) Angenent et al. (1995), Plant Cell **7**, 1569-1582; (3) Pnueli et al. (1994a), Plant Cell **6**, 163-173; (4) Pnueli et al. (1994b), Plant Cell **6**: 175-186; (5) Kelly et al. (1995), Plant Cell **7**, 225-234; (6) Rottmann et al. (1993) J.Cell.Biochem.Suppl. 17B:23; (7) Southerton et al. (1993) J.Cell.Biochem.Suppl. 17B:24; (8) Rutledge et al. (1993) J.Cell.Biochem.Suppl. 17B:45; (9) Tandre et al. (1993) J.Cell.Biochem.Suppl. 17B:16

HARDWOODS

MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS

MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS

TECHNICAL PROGRAM REVIEW
February, 1995 - February, 1996

Project Title: MASS CLONAL PROPAGATION OF GENETICALLY
IMPROVED AND ENGINEERED HARDWOODS
Project Code: HRDWD
Project No.: F011
Division: Chemical and Biological Sciences
PAC: Forest Biology
Project Staff: Faculty: J. Cairney, J. Pullman. (R. J. Dinus)
Staff: K. Floyd (S. Chang, C. Stephens)
FY95-96: $155 579
Long Range Research Needs: Develop an assured supply of competitive virgin fiber.
Increase growth to maximize yield per acre per year.
Improve survivability of planted species.
Improve fiber characteristics to match product and process requirements.

PROGRAM OBJECTIVE:

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

SUMMARY OF RESULTS:

Established new Cottonwood hybrids in culture (*P. tremuloides* x *deltoides*)

Transformation of a formerly recalcitrant clone of *Populus deltoides* been achieved as
confirmed by,
1. Kanamycin resistance of transformed plants
2. Biochemical reaction revealing the presence of the GUS enzyme in transformed tissue
3. PCR amplification of an appropriately sized DNA fragment using GUS primers

Transformation protocol extended to transferring in the iaaM (auxin) gene to *Populus
deltoides* and potential transformants have been identified.

Transformation of Tobacco (a model plant) by with the *iaaM* gene has been confirmed by
PCR using *iaaM* primers.

MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS
PROJECT UPDATES

John Cairney
Ron Dinus
Shujun Chang
Camille Stephens
Karen Floyd
Bobbie Johns

Transformation of Cottonwood with the uidA (GUS) Reporter gene.

Project Update:

Earlier results showed that Cottonwood clones, putatively transformed with a vector encoding Kanamycin resistance and GUS genes and survived Kanamycin selection and gave a strong blue stain in a GUS reaction. As a final confirmation that transformation had been achieved we have conducted PCR using Cottonwood chromosomal DNA and primers designed from the sequence of the GUS gene. Chromosomal DNA was isolated from transformants using the protocol of Doyle and Doyle (BRL Focus 12(1), 1990) and the integrity was checked by running EcoR1 cut and uncut DNA. We were able to generate a DNA fragment of 1.2kb, the size expected given the location of the PCR primers. The PCR conditions were; 250ng DNA, 50pmol of each primer, 1mM MgCl$_2$, 0.25mM dNTPs, 1 unit Taq Polymerase, the cycle conditions were: 95°C, 5 min.; (95°C, 1 min.; 45°C, 1 min.; 72°C, 1 min.) 35 cycles; 72°C 5 min.. This result adds molecular proof to the antibiotic and biochemical evidence that Populus deltoides clone C175 has been successfully transformed.

Transformation of Tobacco with iaaM gene

Project Update:

Using established protocols the iaaM (auxin) gene form Agrobacterium was transferred into Nicotiana tabacum var. xanthin. This work was complementary to the Cottonwood iaaM transformation experiments (below) and was intended to evaluate the effect of iaaM overexpression and use this knowledge in the design of transformation experiments with trees. Despite initial concerns over the efficiency of gene transfer, many transformants were obtained in the this series of experiments. Large, healthy-looking plantlets were generated, a striking feature of which is the abundance of roots growing over almost the entire length of the shoots. We selected several putatively transformed Tobacco plants and isolated chromosomal DNA. Using primers designed to the iaaM gene we conducted a series of PCR experiments. Using conditions we generated a DNA fragment of 0.5kb, the size expected for the amplification from the iaaM gene. The PCR conditions were; 200ng DNA, 70pmol of each primer, 1mM MgCl$_2$, 0.25mM dNTPs, 1 unit Taq Polymerase, the cycle conditions were: 95°C, 5 min.; (95°C, 1 min.; 45°C, 1 min.; 72°C, 1 min.) 35 cycles; 72°C 5 min. Additional bands were observed, presumably from hybridization of primers to endogenous genes. In these experiments we used an annealing temperature of 45°C which may not have prevented less specific hybridization of primers. We are repeating these experiments in an attempt to amplify the transferred gene alone.

These results, show transformation for the transformed tobacco clones which we have evaluated. We do observed differences in size of different plantlets as well as difference in

the number of root hairs observed. We are continuing our evaluation of these transformants by conducting Northern analysis (to determine whether the magnitude of iaaM gene expression can be related to these observations or subsequent growth and development of the transformants, and Southern analysis, to determine whether morphology and/or expression differences can be related to gene copy number.

Transformation of Poplar with iaaM gene

Project Update:

Despite frequent transfers of putative Cottonwood-iaaM transformants they continue to do poorly. We intend culturing them and attempting to regenerate plantlets. At present the few plants we have are too small to sample for PCR.

MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS

Establishment and Propagation of Sweetgum Cultures

MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS

ESTABLISHMENT AND PROPAGATION OF SWEETGUM CULTURES

Gerald Pullman
Shannon Johnson
Cristine Estes
Tom Kraker

SUMMARY

Sweetgum (*Liquidambar styraciflua*) cultures were established using axillary buds and shoot tips from greenhouse origin. The best treatment from Sutter and Barker's 1985 paper, Woody Plant Medium with 1 mg/l BAP and 0.05 mg/l NAA, was utilized. One hundred percent representation of sterile proliferating cultures was achieved out of the five genotypes tested. A second experiment tested the best treatments from Sommer and Brown's 1980 paper in an attempt to initiate Sweetgum embryogenic callus from three different families.

METHODS AND MATERIALS

<u>Experiment 1</u>   Five genotypes were selected to test the proliferation of axillary buds and shoot tips. These included two Union Camp rooted cuttings (UC1 and UC6) and three International Paper (IP) grafts (7A, 7B and 7C). All had been maintained under greenhouse conditions. All clones were from selected Sweetgum trees. The IP selections are under current evaluation by the North Carolina State Hardwood Cooperation.

Stems were cut from the seedlings and grafts in the greenhouse followed by removal of the leaves. They were wrapped in wet paper towels and transported to the lab. The stems were cut into sections at each node, yielding sections with a node on one end only. One shoot tip section was also cut from each stem. Stem and shoot tip sections from the same stem were shaken in 1% Tween 20 for 10 minutes followed by three rinses in sterile water. The stem sections were then shaken in a 20% (v/v) commercial bleach solution for 20 minutes followed by five rinses in sterile water. The stem sections were then cut to yield node pieces of 0.5 cm while the shoot tips were left whole but cut down to 1 - 1.5 cm pieces. These cut pieces were placed into WNAA3 (see Table 1) antibiotic solution and shaken overnight followed by three rinses in sterile water.

Twenty ml of WPM containing 1 mg/l BAP and 0.05 mg/l NAA (medium 494, see Table 1) was placed in deep (100 x 20 mm) petri dishes. The node and shoot tip sections were placed horizontally on the medium. Medium 494 contained antibiotics for the first 28 days of culture (either 250 mg/l cefotaxime or 500 mg/l carbenicillin) to help prevent

contamination. The explants were maintained at 25° ± 1°C, under cool white fluorescent lights with 16 hours/day illumination and were subcultured every 28 days.

<u>Experiment 2</u>   Three Sweetgum families (A, B and C) were supplied from Westvaco Corporation, Summerville, SC. Four week old germinated seedlings were sent through the mail wrapped in moist towels. The seedlings were stored at 4°C for an additional nine days before being sterilized for culture. Ten seedlings from each of the three families were sterilized in the following manner. Leaves and roots were removed and discarded leaving 2.5-3.0 cm hypocotyl sections. The hypocotyl sections were rinsed in running, cold tap water for ten minutes. Sections were then agitated for ten minutes in 10% Liquinox plus two droppers of Tween 20/l. Sections were rinsed with running, cold tap water for another 30 minutes before being placed into a 10% (v/v) commercial bleach solution for ten minutes. Finally, sections were rinsed three times in sterile water.

The outer 1 - 2 mm of the hypocotyl sections were removed and the resulting sections were cut into five pieces approximately 0.5 cm long. Two different media were tested (Table 2.). Medium 459 contained 0.1 mg/l NAA and 0.5 mg/l BAP while medium 460 contained 1.0 mg/l NAA and 0.5 mg/l BAP. Each plate had five hypocotyl pieces coming from one seedling. Also, a student working on a class project tested a node section from family C on medium 494 used in experiment one.

The cultures were maintained at 25° ± 1°C, under cool white fluorescent lights with 16 hours/day illumination and were subcultured once a month. After two months cultures with ample callus were transferred to no hormone liquid medium 458 (Table 2.) in an attempt to develop embryogenic suspension cultures. The remaining explants with adventitious shoot primordia went to solid no hormone medium 457 (Table 2.) to encourage shoot elongation.

RESULTS

<u>Experiment 1</u>   During the first transfer to fresh medium without antibiotics the segments that had become contaminated were discarded. Several nodes had already broken at that point. After two months many of the broken buds had grown too tall for the deep petri dish so they were transferred to magenta boxes onto the same medium 494. Results after three months are shown below.

| Genotype | # of Explants | | Contamination | | # of Broken Buds | % Buds Broken |
	Total	Clean	#	%		(# Broken / # Clean)
UC1	10	3	7	70	2	67
UC6	10	5	5	50	4	80
7A	40	33	7	18	17	52
7B	40	32	8	20	25	78
7C	25	19	6	24	10	53

<u>Experiment 2</u>   After two weeks the explants were examined for contamination. Results are shown below.

Family	# Contaminated	Total # of Explants	% Contamination	# Clean
A	38	50	76	12
B	30	50	60	20
C	13	50	26	37

Contamination consisted mainly of fungus with a few plates having bacteria. Plates that had one or more contaminated explants were transferred to fresh medium 459 without regard to what medium they had been on. Callus had just begun to form on some of the explants.

After four weeks the explants were examined for callus, adventitious shoot primordia or root production. Results are shown below.

Family	Medium	# With Callus / Total	# With Primordia / Total	# With Roots / Total
A	459	10/10	3/10	0/10
A	460	2/2	0/2	0/2
B	459	16/16	1/16	0/16
B	460	4/4	0/4	0/4
C	459	28/28	12/28	1/28
C	460	9/9	0/9	0/9
Totals:		69/69	16/69	1/69
% Totals:		100%	23%	1.45%

After two months cultures with ample callus were transferred to no hormone liquid medium 458 and the remaining explants with adventitious shoot primordia went to solid no hormone medium 457. The suspension cultures never formed embryoids as stated in Sommer and Brown, 1980. Sommer and Brown frequently found embryoids free from the main callus after having reached a torpedolike stage. The only structures seen freely floating in our suspension cultures were roots. The explants with adventitious primordia had some success. Two shoots from family C and one from A have elongated and continued to stay green. These will be subcultured every month and eventually added to our stock of Sweetgum cultures.

The class project of the student was actually very successful. The node section from family C has continued to develop new shoots without being transferred for over three months. This family can be added to the five other genotypes in culture coming from experiment one.

DISCUSSION

<u>Experiment 1</u>   Using this protocol we have secured clean, tissue culture material for each of the five genotypes tested. With these cultures now in hand, the next step will be to test protocols from the literature concerning plant regeneration from adventitious buds from leaves. Success in this plant regeneration area would eventually lead to genetic engineering trials by the Forest Biology team.

<u>Experiment 2</u>   The effort to obtain Sweetgum embryogenic callus from young seedlings did not prove to be successful. The hypocotyl sections did, however, give rise to adventitious shoot primordia and roots. A few primordia went on to elongate into shoots and will continue to be transferred. The student who cultured the meristematic portion of a Family C young seedling achieved great success on Sutter and Barker's best treatment as used in Experiment 1.

Experiment 1 used older tissue while Experiment 2 used young seedlings. When testing medium 494 the younger tissue responded more rapidly, with continuously breaking buds. The older tissue has responded more slowly with not much multiple/continuous bud breakage.

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Components	Media (mg/l)	
	494	WNAA3
NH_4NO_3	400	2,000
K_2SO_4	990	990
$MgSO_4 \cdot 7H_2O$	370	370
KH_2PO_4	170	170
$Ca(NO_3)_2 \cdot 4H_2O$	556	1,650
$CaCl_2 \cdot 2H_2O$	96	96
H_3BO_3	6.2	6.2
$MnSO_4 \cdot H_2O$	22.3	22.3
$ZnSO_4 \cdot 7H_2O$	8.6	8.6
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.25	0.25
$FeSO_4 \cdot 7H_2O$	27.8	27.8
Na_2EDTA	37.3	37.3
Sucrose	20,000	20,000
myo-Inositol	100	100
Casein hydrolysate	--	500
Thiamine•HCl	1.0	1.0
Pyridoxine•HCl	0.5	0.5
Nicotinic acid	0.5	0.5
Glycine	2.0	2.0
NAA	0.05	--
BAP	1.0	--
Cefotaxime	--	500
Tetracycline	--	50
Rifampcin	--	15
Gelrite	2,500	--
pH	5.2	5.7

Table 1. Medium components for establishment of Sweetgum from the greenhouse.

Components	Media (mg/l)			
	457	458	459	460
NH_4NO_3	1,000	1,000	1,000	1,000
KNO_3	1,000	1,000	1,000	1,000
$MgSO_4 \cdot 7H_2O$	71.7	71.7	71.7	71.7
KH_2PO_4	300	300	300	300
$Ca(NO_3)_2 \cdot 4H_2O$	500	500	500	500
KCl	65	65	65	65
KI	0.6	0.6	0.6	0.6
H_3BO_3	2.0	2.0	2.0	2.0
$MnSO_4 \cdot H_2O$	6.06	6.06	6.06	6.06
$ZnSO_4 \cdot 7H_2O$	4.0	4.0	4.0	4.0
$Na_2MoO_4 \cdot 2H_2O$	0.025	0.025	0.025	0.025
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025	0.025
$FeSO_4 \cdot 7H_2O$	27.8	27.8	27.8	27.8
Na_2EDTA	37.25	37.25	37.25	37.25
Sucrose	40,000	40,000	40,000	40,000
myo-Inositol	100	100	100	100
Thiamine•HCl	1.0	1.0	1.0	1.0
Pyridoxine•HCl	0.1	0.1	0.1	0.1
Nicotinic acid	0.1	0.1	0.1	0.1
NAA	--	--	0.1	1.0
BAP	--	--	0.5	0.5
Bacto Difco Agar	8,000	--	8,000	8,000
pH	5.7	5.7	5.7	5.7

Table 2. Medium components for initiation of embryogenic callus from Sweetgum.

MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS

MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS

Ronald J. Dinus
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GREENHOUSE CLONE BANK

Maintenance of cottonwood clones in the greenhouse continues as per past reports. Nine elite clones, obtained from James River Corporation, were added to the greenhouse collection in late winter. Four were used to replace clones used in research over the past several years. Five are new to the program.

Also added were 10 hybrid clones (Populus trichocarpa X deltoides). These were provided by cooperators at the University of Washington and Washington State University. Included are a number of triploid and sterile clones for use in transformation research. Permits for field testing transgenic plants derived from such clones can obtained with far less difficulty than for nonsterile trees.

Significant numbers of ramets died during the record heat of summer. Several valuable clones were lost. Research on culture establishment and plant regeneration via internode and leaf section systems, however, was initiated well before the mortality was incurred. Indeed, representatives of affected clones have been established in and are being maintained in culture (See below for section on: ESTABLISHING ELITE CLONES IN CULTURE). Regeneration frequencies have been high, and the needed number of replacements are being conditioned for movement to the greenhouse.

CULTURE ESTABLISHMENT AND REGENERATION OF ELITE CLONES

Internode System

Research on establishment and multiplication of cultures as well as regeneration of plants from such cultures was renewed. A large experiment was executed to establish cultures of the newly acquired hybrid clones for use in future transformation research. Procedures for explant processing, media preparation, and response measurement were performed as per Stephens et al. (1994).

All 10 hybrid clones were tested against four treatments, considered applicable on the basis of previous experience with

<u>P. deltoides</u> clones at IPST and with similar hybrids (Wang 1995, Personal Communication). Treatments were: culture on control or basal medium (no growth regulators), direct placement on shoot induction medium, and transfer to shoot induction medium after exposure to callus inducing medium for one and four days. Five internode sections were used per petri plate or replication. Numbers of replications varied among clones and treatments in accordance with explant availability. The number of replications per clone and treatment combination, typically, was six, although numbers ranged from 1 to 10.

Explants forming callus, primordia, and shoots were counted at periodic intervals through 91 days in culture. Shoot harvest could have continued beyond the 91st day, but the trial was terminated at that time to make way for other research. Also, sufficient shoots had been harvested by that time for evaluation of rooting performance.

For statistical analyses, results were summarized as percentages of explants producing callus, primordia, and shoots. Productivity was also evaluated in terms of numbers of shoots produced per explant. Cumulative results obtained through 91 days were subjected to analyses of variance for the completely random design. Analyses tested overall clone, treatment, and interaction effects as well as treatment effects within individual clones.

Overall analyses of variance indicated that clone, treatment, and interaction effects were significant for all variables. Treatment effects within clones generally were also significant regardless of variable. Treatments did not produce differential effects for a few clones, ie., response was uniformly high or low to all treatments. As an example, clone 17 X 50 formed callus, primordia, and shoots at high frequency regardless of treatment (Tables 1 through 3). Yields of shoots per 17 X 50 explant, however, were more variable (Table 4).

Table 1. Mean percentages of _Populus trichocarpa X deltoides_ internode explants forming callus after culture on basal medium (Control), shoot induction medium (SIM), and shoot induction medium after exposure to callus induction for one (CIM-1) and four days (CIM-4). Observations are cumulative percentages through 91 days of culture.

Clone No.	Control	SIM	CIM-1	CIM-4	Clone Mean
17 x 50	100	100	100	100	100
22 x 86	79	100	100	100	95
23 x 99	71	100	100	100	93
23 x 106	60	100	98	100	90
24 x 110	100	100	100	98	100
24 x 112	71	100	100	100	93
50 x 184	57	100	100	100	89
52 x 226	75	100	100	100	94
184 x 402	70	100	100	100	92
184 x 405	93	100	100	100	98
Treatment Mean:	78	100	100	100	94

Table 2. Mean percentages of _Populus trichocarpa X deltoides_ internode explants forming primordia after culture on basal medium (Control), shoot induction medium (SIM), and shoot induction medium after exposure to callus induction for one (CIM-1) and four days (CIM-4). Observations are cumulative percentages through 91 days of culture.

Clone No.	Control	SIM	CIM-1	CIM-4	Clone Mean
17 x 50	100	100	100	100	100
22 x 86	79	100	100	96	94
23 x 99	57	100	100	98	89
23 x 106	50	100	98	94	85
24 x 110	79	100	96	88	90
24 x 112	43	100	94	92	82
50 x 184	36	100	100	100	84
52 x 226	75	93	79	100	87
184 x 402	50	100	95	98	86
184 x 405	86	100	98	100	96
Treatment Mean:	65	99	96	96	89

Table 3. Mean percentages of <u>Populus trichocarpa X deltoides</u> internode explants forming shoots after culture on basal medium (Control), shoot induction medium (SIM), and shoot induction medium after exposure to callus induction for one (CIM-1) and four days (CIM-4). Observations are cumulative percentages through 91 days of culture.

Clone No.	Control	SIM	CIM-1	CIM-4	Clone Mean
17 x 50	100	100	100	98	100
22 x 86	71	100	74	52	74
23 x 99	29	64	76	74	61
23 x 106	40	53	68	64	56
24 x 110	71	93	88	75	82
24 x 112	36	50	57	77	55
50 x 184	21	64	76	98	65
52 x 226	75	90	64	58	72
184 x 402	70	90	65	70	74
184 x 405	36	57	81	84	64
Treatment Mean:	55	76	75	75	70

Table 4. Numbers of shoots per <u>Populus trichocarpa X deltoides</u> internode explant after culture on basal medium (Control), shoot induction medium (SIM), and shoot induction medium after exposure to callus induction for one (CIM-1) and four days (CIM-4). Observations are cumulative percentages through 91 days of culture.

Clone No.	Control	SIM	CIM-1	CIM-4	Clone Mean
17 x 50	2.6	9.3	7.8	5.7	6.4
22 x 86	3.7	4.8	4.5	3.5	4.1
23 x 99	1.7	3.4	4.7	2.7	3.1
23 x 106	1.4	2.5	2.8	2.2	2.2
24 x 110	2.5	3.9	2.8	2.2	2.8
24 x 112	0.6	1.5	2.8	2.8	1.9
50 x 184	0.8	1.4	4.0	4.6	2.7
52 x 226	4.2	7.2	2.4	3.2	4.2
184 x 402	1.4	1.6	2.8	3.5	2.3
184 x 405	0.6	1.1	3.4	2.3	1.8
Treatment Mean:	2.0	3.7	3.8	3.3	3.2

Although these data represent only one trial, all 10 hybrid clones performed as well as model clones used in our laboratory, and generally surpassed elite <u>P. deltoides</u> clones tested in past trials. Two clones, 17 X 50 and 24 X 110 seem especially worthy of further testing, and eventual use in transformation research.

As indicated above, shoots were counted and harvested periodically through the 91st day in culture. As shoots were harvested, they were moved through the rooting protocol used routinely with our Leaf Section System. An effort was made to capture approximately 60 shoots per clone. Rooting frequencies (Table 5) were more than acceptable for maintenance and multiplication.

Table 5. Rooting frequencies of shoots harvested from internode cultures of 10 _Populus trichocarpa X deltoides_ clones.

Clone No.	No. of Shoots	Percent Rooting
17 X 50	64	83
22 X 86	84	76
23 X 99	40	97
23 X 106	56	50
24 X 110	64	78
24 X 112	67	82
50 X 184	56	100
52 X 226	48	88
184 X 402	56	88
184 X 405	24	100

As a next step, responsiveness of explants from culture will be evaluated for all clones. The two more responsive clones noted above, however, will be emphasized. Suitability for multiplication of cultures and transformation will be assessed via both the internode and leaf section protocols.

Leaf Section System

Responsiveness of leaf sections from the 10 hybrid clones was also evaluated in a small scale, preliminary trial. Leaves were collected from the greenhouse in early spring. Since only three ramets were available per clone, collection of leaves suitable for use as explants proved difficult. In some cases, leaves were used that were older than considered optimal. For these reasons, the trial must be considered preliminary.

The trial used the standard Leaf Section System protocol. Numbers of leaf sections and petri plates or replications varied among clones in line with explant availability. From 5 to 11 leaf sections were used per replication, with numbers of replications ranging from 4 to 8 per clone. Typically, replications numbered seven per clone.

Explants forming callus, primordia, and shoots were counted at several intervals through 81 days after culture. Shoot formation and elongation appeared to have ceased by that time, and the experiment was terminated. Given preliminary nature of the trial,

results were not subjected to statistical analysis.

Observations indicated that callus formed rapidly on all explants that responded to treatment during course of the experiment. Indeed, callus was evident on responsive explants 7 to 14 days after start of culture. Such performance is most encouraging in that P. deltoides clones noted for shoot productivity in this protocol typically behave in this manner. This behavior also infers indirectly that more P. trichocarpa X deltoides explants would have responded with callus and shoot formation if the test had included only leaves of the most appropriate ages. That most clones formed callus at moderate to high frequency and also produced some shoots (Table 6) is also encouraging. That is, perhaps half or more of the clones have potential for multiplication and transformation via the leaf section system.

Table 6. Callus and shoot production of Populus trichocarpa X deltoides leaf sections in the IPST Leaf Section System protocol. Observations are cumulative percentages through 81 days of culture.

Clone No.	Explants (No.)	Percentages of Callus	Explants Forming Shoots
17 x 50	36	92	11
22 x 86	57	84	12
23 x 99	39	72	8
23 x 106	57	56	1
24 x 110	38	76	13
24 x 112	42	90	33
50 x 184	50	64	10
52 x 226	37	70	11
184 x 402	80	62	5
184 x 405	64	45	11
Clone Mean:	N/A	71	12

As noted above for the related internode trial, efforts will be undertaken in the near future to test suitability of the most responsive clones for multiplication, maintenance, and transformation via both the leaf section and internode systems.

GENETIC TRANSFORMATION

Transformation of Cottonwood with the uidA or GUS Reporter Gene

Two experiments with the GUS reporter gene (Trans 94-2 and -4) were completed in late spring. Results through early spring were presented during the Annual Program Review, March 21-22, 1995. Transformation had been accomplished, and several transgenic plants had been recovered. Transformation frequencies, however, were rather low, and regeneration was hindered by the antibiotic used to select transformed cultures.

Two additional trials (Trans 94-5A and -5B) have since been executed in an attempt to remedy these shortcomings. Each of these experiments sought to raise transformation frequencies by adjusting Agrobacterium tumefaciens (At) exposure times and concentrations, and by delaying exposure to selective medium. Two contrasting exposure times (30 and 120 min) and concentrations (1 X 10**7 and 1 X 10**10 cfu/mL) were evaluated. Delayed selection, ie., culture on nonselective medium for 14 days before exposure to the selective antibiotic, was intended to compensate for the extreme sensitivity of cottonwood to the antibiotic. We theorized that delayed selection would allow the one or few transformed cells to multiply, differentiate, and develop to an extent sufficient to withstand the debilitating effects of the antibiotic and the numerous dead and dying nontransformed cells around them. Except for incorporation of these new treatments, experimental designs and protocols were similar to those of earlier trials.

Results from three of the four trials described above were presented at the Southern Forest Tree Improvement Conference in June. A copy of the published paper is included herewith as Attachment 1. Findings from all four trials were presented at the International Poplar Symposium, University of Washington, in August. A copy of the published abstract is provided as Attachment 2.

In sum, exposure to At for 120 min proved more effective than for 30 min, and the higher concentration produced more transformants than the lower one. Although longer exposure time and higher concentration inhibited shoot production to some extent, this treatment combination, when used in conjunction with delayed selection, produced the highest frequency of transformed cultures (13 percent). Delayed selection generally improved transformation frequencies, regardless of At exposure time or concentration, and did not noticeably increase occurrence of escapes or chimeric transformants.

Subsequent efforts have focused on regenerating plants from cultures showing expression of the GUS gene at the end of lengthy culture periods (91 or more days), and on assaying derived plants

for continued expression. To date, plants have been regenerated from 50 percent of these cultures. A total of 43 plants have been regenerated, rooted, and thereby made available for assay. Leaves from all such plants were assayed after 3 to 6 months of growth, and leaves from 34 or 79 percent of the plants showed GUS expression. Expressed on a per explant basis, treatments examined in the most recent experiment (Trans 94-5B), including delayed selections, yielded a transformed plant frequency of 10 percent.

Next steps involve regenerating and assaying more plants from more of the transformed cultures. Also, a journal article will be prepared as quickly as additional confirmation of transformation is obtained. That is, a subsample of plants will be assayed for presence of the GUS gene via either PCR or Southern blotting methods.

Transformation of Cottonwood with the iaaM gene

Two experiments with the iaaM gene were terminated in late spring. Results through early spring were summarized for the Annual Program Review, March 20-21, 1995. Several transgenic cultures were obtained, but transformation frequencies were low and efforts to regenerate plants were not successful. Transgenic cultures, however, are being maintained in the event that regeneration becomes possible and/or for use in studying methods for activating the controllable heat shock promoter. During the interim, as evident from the foregoing efforts, most resources were devoted to refining the transformation protocol by working with the GUS gene.

More recently, an experiment (Trans 95-3) capitalizing on improvements gained through trials with the GUS gene was initiated. The At exposure time and concentration found best in the GUS trials gene were employed in this new iaaM test. The practice of delaying selection was also incorporated. In addition, efforts were made to remedy past problems with residual At contamination. The strain of At used with the iaaM gene is especially aggressive, and significant numbers of cultures and explants have been lost in the past. As a result, a washing and antibiotic (cefotaxime) rinsing step (Wang 1995, Personal Communication) was added to our protocol.

Treatments included: a positive control (-At-Selective Antibiotic (kanamycin (K)), an At virulence check (+At-K), immediate selection via transfer directly to medium containing K, and delayed selection for 14, 17, and 21 days. Other than addition of the delayed selection treatments and wash/rinse step, experimental design and protocol were the same as in past trials.

Preliminary results (Table 7) indicate that the extended At exposure time and somewhat heightened concentration, coupled with delayed selection, appear to have increased numbers of putative transformants over those observed at the shorter exposure times (5 and 30 min) and lower concentrations used in previous trials.

Table 7. Transformation of cottonwood clone C175 with the iaaM gene (Trans 95-3) (1). Observations are cumulative percentages of leaf sections forming callus, primordia, and shoots as well as exhibiting residual _Agrobacterium tumefaciens_ contamination through the 63rd day in culture.				
Treatment	Percentages of leaf sections having			
	Callus	Primordia	Shoots	Contamination
Control (-At-K)	100	100	27	N/A
Virulence				
Check (+At-K)	100	100	33	13
Selection (+At+K)				
Immediate	57	43	0	50
Delayed, 14 days	74	46	9	54
" , 17 "	89	70	10	31
" , 21 "	93	73	17	57
(1) _Agrobacterium tumefaciens_ strain and vector: ASE (pTiT37SE) containing pMON604; Exposure time = 120 min at concentration of 1 X 10**8 cfu/mL.				

More importantly, development clearly is progressing farther and faster with increasing time to placement on selective medium. That is, percentages of explants forming shoots tend to be greater when selection is delayed by 21 days. Most aspects of development, however, are improved by delayed selection, regardless of time to placement on selective medium. Evidence that these cultures and/or shoots are indeed transgenic and not merely escapes will not be available until presence of the gene is confirmed by PCR assays.

Shoots have been and are being harvested, rooted, and transferred to growth medium. Selection with K is being applied in both the rooting and growth media to reduce the number of escapes. Shoots surviving and exhibiting active growth after three months will be subjected to PCR assays to confirm presence of the iaaM gene. Shoots confirmed as transgenic will be multiplied, and used in subsequent tests of methods for activating the heat shock promoter. Effects of iaaM gene expression in young plants will then be investigated.

Residual At contamination, regrettably, remains a problem. Periodic washings and antibiotic rinses will be continued. Should this not be successful, future research might best be postponed until the plasmid bearing the iaaM gene can be moved into another At strain. Transfer into a strain such as that used in earlier trials with the GUS gene may be the only way to prevent the interference and loss occasioned by use of the present strain.

Transformation of Tobacco with iaaM gene

Two transformation experiments (Trans 95-4 and -5) are underway with tobacco. The purpose of these trials is to produce tobacco plants transgenic for the iaaM gene and heat shock promoter. In turn, transgenic plants will be used to develop and test environmental conditions needed to activate the promoter and gene, and to determine what traits are most likely to be affected by gene expression. Trial runs with tobacco were viewed as a means of easing the way for eventual handling of transgenic cottonwood.

The At strain employed in these trials was the same as that used with cottonwood. The tobacco cultivar was (Nicotiana tabacum var. xanthin). Standard laboratory media and protocol were employed for culture and transformation, respectively (Chang 1995, Personal Communication). The washing and cefotaxime rinsing step noted above was employed to minimize residual At contamination in both experiments.

Trans 95-4 consisted of 3 petri plates or replications of a positive control (-At-K) and a test population (+At+K) of 20 replications. Each replication contained five leaf sections. Numbers for Trans 95-5 were control group, 6 replications and test population, 34 replications. Control groups will be used to monitor nature and rates of development and to regenerate nontransformed plants for use in any repeat trials as well as in efforts to activate the promoter and gene.

Observations to date indicate rapid development in both control populations; shoots formed quickly and on 100 percent of the explants in both trials. Test populations appear to have developed somewhat slower and to a lesser extent; ie., fewer explants have shoots, shoots tend to be fewer per explant, and fair numbers of explants appear unhealthy. In Trans 95-4, only 81 percent of the explants have produced shoots to date. The comparable number for Trans 95-5 is 72 percent.

Although the experiments have been in progress a relatively short time, these results suggest that the selective medium is inhibiting development and that some proportion of shoots formed to date may be transformed. Accurate assessment awaits observation of their performance on selective rooting and growth medium, and subsequent PCR assay.

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ACKNOWLEDGEMENTS

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ATTACHMENT 1

AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION OF POPULUS DELTOIDES LEAF SECTIONS

Ronald J. Dinus, Camille J. Stephens, and Shujun Chang[1]

Abstract. Several factors, including Agrobacterium tumefaciens (At) exposure times and concentrations, were varied in efforts to increase transformation efficiency. Leaf sections of Populus deltoides clone C175, collected from shoot cultures, were inoculated with At strain LBA4404 carrying binary vector pBI121. Included in the vector were the selectable marker gene (NPTII) for kanamycin (K) resistance and the reporter gene (uidA) for beta-glucuronidase production. Transformants were identified by selection on medium containing 50 mgK/L and confirmed by histochemical staining for uidA expression. Exposure to At for 120 min proved more effective than shorter times, and elevated concentrations gave more transformants than lower ones. Long exposure times and high concentrations, however, tended to reduce shoot formation. Selection of putative transformants with 50 mgK/L proved workable, but this level clearly inhibited regeneration. The selection process was therefore modified to include culture on nonselective medium for 14 days before transfer to selective medium. This gave higher transformation frequencies than otherwise obtained, apparently a result of transformed calli enlarging and organizing sufficiently to develop on selective medium.

Keywords: Cottonwood, Poplar, Organogenesis, uidA Gene, Gene Transfer.

INTRODUCTION

Populus species and hybrids are among the fastest growing and most commercially important forest trees in the world. Eastern cottonwood (P. deltoides) (Pd) is especially noted for rapid growth and desirable pulping and papermaking properties. Significant genetic improvement has been obtained via classical selection and breeding, and the species is regenerated and planted vegetatively. More rapid and specific improvement, however, may be obtained by insertion of genes for traits not available in the species.

Genetic transformation has become almost routine with a variety of dicotyledonous plants, including a number of Populus species and hybrids. Indeed, the genus has proven to be a model for insertion of genes having commercial value (Chandler 1995). Much of this research, however, was performed with taxa other than Pd or its hybrids (e.g. Fillatti et al. 1987). Transformation has also been accomplished with hybrids between Pd and other species; e.g, P. trichocarpa (Parsons et al. 1986, De Block 1990, Wang et al. 1994) and P. nigra (Charest et al. 1992, Devantier et al. 1993). Against this background, we sought to devise a transformation protocol for Pd, with the intent of extending it from clones noted for ease of manipulation in culture to elite clones of commercial value.

In our earlier work (Stephens and Dinus 1994), a gene for enhanced auxin synthesis (Klee et al. 1987) was inserted into a model Pd clone (C175). Transformation frequencies, however, were low, and transgenic plants were not recovered. Accordingly, research was continued with a benign marker gene, the

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uidA reporter gene, to improve protocol efficiency. The present report describes results from three experiments in which several factors hypothesized as important to transformation efficiency were evaluated. Included were: preincubation treatments (Fillatti et al. 1987, Tsai et al. 1994, Confalonieri et al. 1994), At exposure time, and At concentration (Confalonieri et al. 1994). Effectiveness of two antibiotics for clearing cultures of residual At contamination and of culture on nonselective medium for a short time before transfer to selective medium were also tested. Adjustment of these several factors resulted in increased transformation frequencies and recovery of transgenic calli, primordia, and shoots.

MATERIALS AND METHODS

Plant Materials. Three experiments were conducted to evaluate factors influencing genetic transformation and regeneration of transgenic plants from eastern cottonwood clone C175 (Dr. S.G. Ernst, University of Nebraska-Lincoln). This model clone, easily manipulated in culture, was maintained in continuous shoot cultures to supply leaf explants over the long term.

Transformation and Regeneration. A leaf section system developed by Uddin et al. (1990), and modified for transformation by Shorter (1991) was used for transformation and regeneration. Procedures used in research reported here have been described by Dinus (1992) and Dinus and Stephens (1994). Five leaf sections were used per petri plate or replication; numbers of plates varied among experiments and treatments as indicated below.

Transformation was done with At Strain LBA4404 containing the binary vector pBI121. Included in the vector were the selectable marker gene (NPTII) for kanamycin (K) resistance coupled to the NOS promoter and the uidA reporter gene linked to CaMV35S (Clonetech Laboratories Inc.). At was cultured in YM broth (Lin 1994) at 30°C for three days, sedimented by centrifugation at 2500 rpm for 5 min, resuspended in standard leaf section medium, and diluted to the concentrations used for transformation.

Antibiotics. Selection of putative transformants was done on medium containing 50 mgK/L. Earlier research with C175 (Shorter 1991) showed that K levels as low as 30 mg/L halt development of nontransformed C175 leaf sections. In the present research, 50 mg/L was used as a safety margin and as recommended by Clonetech Laboratories Inc. Lethal dose assays (Shorter 1991) showed that carbenicillin (CA) concentrations as high as 500 mg/L did not harm C175 leaf sections, and were reasonably effective at clearing cultures of residual At. This concentration was used in the present research until questions arose about efficacy of CA. In response to such questions, cefotaxime (CE) was also evaluated in a lethal dose assay (Dinus et al. 1995). At growth was stopped by 250 mg/L or more, without apparent detriment to C175 leaf sections. Utility in C175 transformation trials, however, was not evaluated until experiments reported herein. All antibiotics were obtained from Sigma Chemical Co., St. Louis, MO. Other changes made to the aforementioned protocols are described below in the context of individual experiments.

Identification and Confirmation of Transformation. Callus, primordia, and/or shoots surviving on selective media were counted as putative transformants. Assays for expression of the uidA gene were performed as per the histochemical methods of Jefferson et al. (1987). Small portions of putatively transformed

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calli or leaves were used for assay. Nearly all putative transformants were free of At contamination. The few suspected of being contaminated were rinsed with 70 percent ethanol three times and sterile distilled water twice before assay. When color developed within a few minutes or between plant cells, candidates were not counted as transformed. In sum, plant materials counted as confirmed transformants included only those showing complete expression at the end of the lengthy culture periods noted above. Those showing transient or chimeric expression, or responses due to At contamination, were excluded.

Trial 1. The first experiment evaluated efficacy of the At exposure time (5 min) and concentration (10^9 cfu/mL) used in our earlier research (Shorter 1991). Protocols described above were followed with one exception. Several authors working with Populus hybrids and species have incubated explants for 24-48 hr before exposure to At in order to foster explant growth (Fillatti et al. 1987) or to cull unhealthy explants (Tsai et al. 1994). Accordingly, half of all leaf sections used in this trial, regardless of subtreatment, were incubated on standard leaf section medium for 24 hr in darkness prior to At exposure.

Subtreatments are described below. A control (-At-K) was included to verify that leaf sections developed normally (N = 30). To quantify effects of At and the transformation process on regeneration, leaf sections were exposed to At but not K (+At-K) (N = 110). This treatment also was intended to produce putatively transformed shoots for later selection on shoot growth medium containing K. Results from this latter aspect will be reported elsewhere. A +At+K subtreatment was used to assay yields of transformants resulting from selection immediately after exposure to At (N = 110).

Percentages of leaf sections forming callus, primordia, and harvestable shoots were recorded weekly for the first 63 days of culture and at roughly 3 week intervals through 277 days. Putative transformants were assayed for uidA expression at the end of the trial.

Trial 2. The second experiment tested effects of longer At exposure time (30 min) and a lower concentration (10^8 cfu/mL). As a secondary objective, utility of CE for clearing cultures of residual At contamination was compared to that of CA.

Protocols described above were used with one change; half of the leaf sections, regardless of subtreatment, were cultured on medium supplemented with 500 mgCA/L and half on medium containing 250 mgCE/L. As in the foregoing trial, leaf sections were divided among three subtreatments: -At-K control (N = 30); +At-K (N = 150); and +At+K (N = 150).

Percentages of leaf sections forming callus, primordia, and shoots were recorded weekly for the first several weeks to establish that development was proceeding normally. Observations continued at roughly 6-week intervals thereafter. The trial was terminated after 213 days of culture, when putative transformants from the +At+K subtreatments were assayed for uidA expression.

Trial 3. The third experiment compared effects of varying At exposure times (30 versus 120 min) and concentrations (10^7 versus 10^{10} cfu/mL). Subtreatments used in the aforementioned trials were included, and contrasted

with another that provided for culture on nonselective medium for 14 days before transfer to selective medium (+At+KP). The intent was to allow time for development to start, thereby permitting transformed cells to accumulate and differentiate to an extent sufficient to resist the debilitating effects of K and of the dying cells surrounding them.

Protocols were the same as those used earlier, except for changes described immediately above. Leaf sections were divided among 13 treatment combinations as follows: -At-K (N = 15); +At-K, 4 combinations of exposure times and concentrations (N = 15 per combination); +At+K, 4 combinations (N = 30 per combination); and +At+KP, 4 combinations (N = 30 per combination).

Percentages of leaf sections forming callus, primordia, and harvestable shoots were recorded weekly for the first few weeks, and at roughly four week intervals through the 91st day of culture. The experiment was then terminated, and putative transformants were collected from +At+K and +At+KP treatments for uidA assay and regeneration.

RESULTS AND DISCUSSION

Trial 1. Results from the first trial showed that incubation on standard leaf section medium for 24 hr before exposure to At did not provoke differential responses. Accordingly, results were averaged over all explants given each subtreatment.

In retrospect, this finding is not surprising. Though such practices are used with some frequency (Tsai et al. 1994, Confalonieri et al. 1994), few data are available to substantiate efficacy of this extra step in transformation protocols. Also, effectiveness of such treatments would seem dependent upon their being sufficiently long to ensure that development not only starts but also gets well underway. The time course of development for control leaf sections (Figure 1) shows that callus formation began in the first few days of culture. Primordia, first visible manifestation of organized meristematic centers, however, appeared 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 the first 14 to 21 days of culture. Such treatment also seems best applied after At exposure but before transfer to selective medium. This would permit transformed cells to multiply before dying cells surrounding them interfere with development. This approach was tested in Trial 3; the outcome is described below.

Development on +At-K leaf sections was delayed relative to those given the control treatment (Figure 1, Table 1). Percentages of explants forming callus and primordia eventually reached control levels, but shoot formation and elongation were delayed and reduced relative to controls. Thus, exposure to At and other aspects of the transformation protocol appear disruptive to regeneration.

Development on explants given the +At+K treatment was slow; callus was not evident until the 56th day of culture. Primordia and shoot formation were inhibited (Table 1) even though fair numbers of calli survived and continued to grow. Midway through the trial, a number of leaf sections showing promise were removed from this medium, cultured on nonselective medium to foster

Figure 1: Percentage of Explants from Trial 1 Having Callus (-●-),
Primordia (-■-), and Shoots (-▲-) Through Day 63; A (-At-K) and B (+At-K)
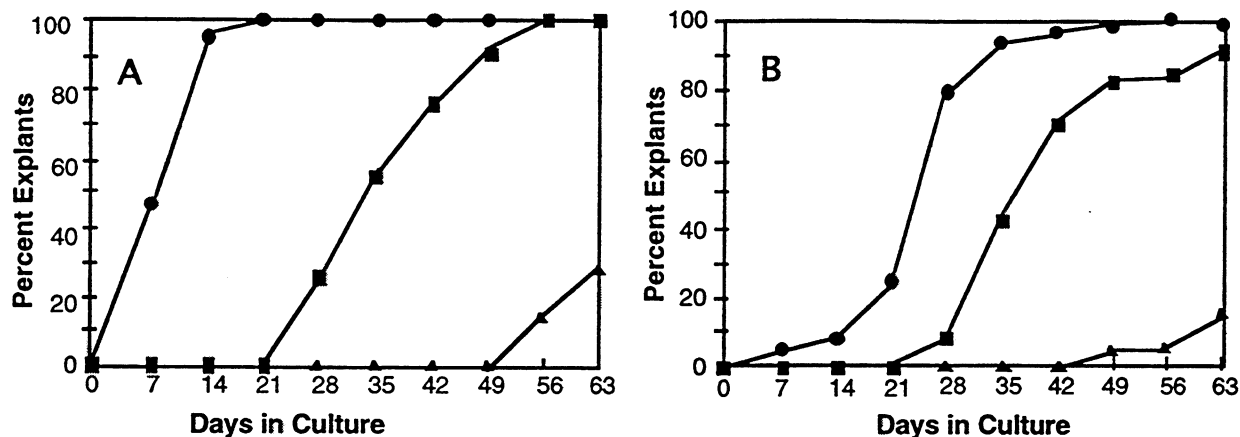

Table 1: Development on C175 leaf sections 277 days after exposure to At (10^9 cfu/mL) for 5 min and cultured on medium supplemented with 500 mgCA/L.

Treatment	Percent of Leaf Sections Forming		
	Callus	Primordia	Shoots
-At-K	100	100	94
+At-K	100	97	9
+At+K	38	0	0

development, and then returned to selective medium. They may not have survived if left on selective medium. As a result, percent of +At+K explants forming callus (Table 1), and therefore percent putative transformants (Table 2), is inflated by roughly 9 percentage points.

As noted above, all +At+K calli that survived through end of the trial were regarded as putative transformants (Tables 1 and 2). Only 5 percent showed expression of the uidA gene, and only 1 percent were rated as confirmed transformants. Thus, this short AT exposure time did not produce high frequencies of lasting transformation despite the relatively high At concentration, a finding in line with other recent investigations in which, depending on species and explant, exposure times ranged from 20 (Confalonieri et al. 1994) to 240 min (Wang et al. 1994).

Trial 2. Findings from the second trial, an effort to increase transformation rates via a longer At exposure time, mirrored those of the first trial. Frequencies of control explants forming callus, primordia, and shoots reached 100 percent quickly, regardless of CA and CE treatment.

Performance of +At-K leaf sections was similar to that of controls (Table 3), except that shoot formation was somewhat lower. Though small, this differential response confirms that At exposure and/or the transformation process reduces regeneration potential. Differences between responses to CA and CE, however, were nominal.

Table 2: Putative and confirmed transformation frequencies as affected by At exposure time (ET) and concentration (Conc).

At ET and Conc. (min. and cfu/ml)		Transformation, Percent of Available Leaf Sections	
		Putative	Confirmed
Trial 1: 5, 10^9		38	1
Trial 2: 30, 10^8,	CA	13	1
	CE	19	1
Trial 3: 91 Days On Selective Medium			
30, 10^7		10	0
10^{10}		37	0
120, 10^7		36	10
10^{10}		27	10
14 Days on Nonselective + 77 Days On Selective Medium			
30, 10^7		27	10
10^{10}		33	10
120, 10^7		43	3
10^{10}		47	13

Explants given the +At+K treatment also formed callus and primordia with some frequency (Table 3), higher than in the first experiment. Shoot formation was also higher. No differences were apparent between responses to CA and CE.

Frequency of putative transformants averaged 16 percent; CA and CE produced similar outcomes (Table 2). All transformants, putative and confirmed, were calli; none of the primordia or shoots surviving on selective medium were transgenic. Only 3 percent of surviving calli showed uidA expression, and only an average of 1 percent were counted as confirmed transformants. These few transformants were distributed equally between CA and CE treatments. Thus, increasing At exposure from 5 to 30 min, and slightly reducing concentration to a level considered desirable by other workers (e.g., Confalonieri et al. 1994) did not raise transformation frequencies above levels noted earlier.

Regardless of subtreatment, differences between CA and CE treatments were minor, thereby confirming that CE does not interfere with regeneration from C175 leaf sections (Table 3). Indeed, when all subtreatments are considered, CE may have had a slight advantage in that numbers of putative transformants were slightly higher than for CA, an outcome possibly associated with lesser interference by residual At.

Residual At contamination was not as severe a problem in this experiment as in our earlier research. Midway through the present experiment, percentages of contaminated explants ranged from 43 to 60 percent, with +At+K explants most affected. Contamination levels were similar on CA and CE medium. With time, however, the margin between antibiotics widened,

Table 3: Development of C175 leaf sections 213 days after exposure to At (10^8 cfu/mL) for 30 min and cultured on media supplemented with 500 mgCA/L or 250 mgCE/L.

Treatment	Percent of Leaf Sections Forming		
	Callus	Primordia	Shoots
Carbenicillin			
−At−K	100	100	100
+At−K	97	94	82
+At+K	13	3	3
Cefotaxime			
−At−K	100	100	100
+At−K	100	99	100
+At+K	19	7	1

particularly in +At+K subtreatments. Within this subtreatment, 57 percent of explants cultured on CA experienced At contamination at one time or another as opposed to only 39 percent of those given CE. Taken together, such findings confirm those from dose/response assays (Dinus et al. 1995), and demonstrate the utility of using CE in the future.

Trial 3. Treatments tested in the third trial yielded definite increases in transformation frequencies (Tables 2 and 4). The trial was terminated after 91 days. Development generally was better than in Trials 1 and 2, and results from them showed little advantage to longer culture periods.

Table 4: Development of C175 leaf sections as affected by varying At exposure times and concentrations after 91 days of culture.

Treatment		Percent of Explants Forming		
		Callus	Primordia	Shoots
At−K: Control		100	100	40
+AT−K: 30 min,	10^7	100	100	47
	10^{10}	100	100	47
120 min,	10^7	100	100	67
	10^{10}	100	100	20
+AT+K: 30 min,	10^7	10	3	0
	10^{10}	37	0	0
120 min,	10^7	36	3	3
	10^{10}	27	10	3
+AT+KP: 30 min,	10^7	27	10	0
	10^{10}	33	10	0
120 min,	10^7	43	17	0
	10^{10}	47	10	0

Control explants formed callus and primordia at frequencies similar to those noted above (Tables 1, 3, and 4). Shoot production was reduced relative

to earlier experiments, but explants were healthy and yields were expected to increase beyond those noted at 91 days.

Leaf sections given the +At-K treatment performed similarly to controls (Table 4). Though somewhat inconsistent across treatments, shoot production tended to decline with increasing exposure time and concentration. Concentration appeared to have the greater effect. The fact that shoot production was not greater highlights the need for At exposure times and concentrations that maximize transformation without reducing regeneration.

Explants given the +At+K treatment formed callus, primordia, and shoots at similar or slightly higher frequencies than in earlier trials (Tables 1, 3, and 4). The overall increase in survival and development suggests that greater At exposure times and concentrations produce higher frequencies of transformation, even though they reduce regeneration potential to some extent.

Culture for 14 days on nonselective medium before transfer to selective medium increased numbers of +At+KP explants forming callus and primordia. Although shoot formation did not increase relative to +At+K subtreatments in this and other experiments, overall development was enhanced, and the improvement appears associated primarily with longer exposure time.

Similar trends were apparent for percentages of putative and confirmed transformants (Table 2), and yields from +At+KP subtreatments were greater than those from +At+K subtreatments in this and the other two experiments. In addition, frequencies varied directly with exposure time and concentration, with longer exposure time having the more pronounced effect. Concerns that +At+KP putative transformants would be largely transient and/or chimeric were not realized. When averaged over all subtreatments, the difference between confirmed and putative transformants was only slightly greater for the +At+KP subtreatments.

Collectively, findings from the third trial indicate that longer exposure times (120 min) and modest concentrations (perhaps, 10^8) offer much promise for raising transformation efficiency without reducing regeneration potential. That the +At+KP treatment tended to foster development and produced higher frequencies of both putative and confirmed transformants further indicates importance of allowing development to start and proceed for some time before challenging putative transformants with selective medium. This procedure apparently allows transformed calli to form and accumulate meristematic centers sufficiently organized to survive and develop.

Clearly, selection with K is far from ideal, and research on transformation would benefit from availability of a more benign marker gene. Increasing public concern about placing genetically altered trees resistant to antibiotics in the environment emphasizes need for such markers. Until such markers are developed, efficient regeneration of transgenic Pd plants will require some further adjustment of K concentrations in selective media. More importantly, treatments similar to the +At+KP tack used here also seem a workable means to circumvent the barrier posed by selection with K. While this approach resulted in slightly more escapes and partial or transient transformants, overall yields of confirmed transformants were greater and obtained in far less time than with other treatments. Trials to reevaluate

the procedure and to test other time periods on nonselective medium are
underway.

Transgenic calli from Trial 3 have been transferred to media designed to
force development of shoots. Also, leaves from cultures containing primordia
and/or shoots have been harvested for multiplication/regeneration on standard
leaf section medium. Shoots have been rooted, and are being multiplied via
the leaf section protocol. These materials will be used to again verify
transformation via histochemical and polymerase chain reaction assays for uidA
gene expression and presence, respectively, and to check for any abnormalities
in morphology and growth.

Results are also being used to effect transformation with and to study
expression of a gene for enhanced auxin synthesis (Klee et al. 1987) in Pd
clone C175, and to extend transformation to elite clones of commercial value.

ACKNOWLEDGEMENTS

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ATTACHMENT 1

Dinus, R.J., C.J. Stephens, and Shujun Chang. 1995. *Agrobacterium tumefaciens*-mediated transformation of *Populus deltoides* leaf sections. pp 210-219, In: Procs. Southern Forest Tree Improvement Conf., June 20-22, 1995, Asheville, NC.

ATTACHMENT 2

Agrobacterium tumefaciens-Mediated Transformation

of Eastern Cottonwood (Populus deltoides)

Ronald J. Dinus, Camille J. Stephens, and Shujun Chang

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Abstract. Several factors, including Agrobacterium tumefaciens
(At) exposure times and concentrations were varied to increase
transformation efficiency.   Leaf sections of Populus deltoides
clone C175, collected from shoot cultures, were inoculated with At
strain LBA4404 carrying binary vector pBI121. The vector contained
the selectable marker gene (NPTII) for kanamycin (K) resistance and
the reporter gene (uidA) for beta-glucuronidase production.
Transformants were identified by selection over three or more
months of culture on K-containing medium, and confirmed by
histochemical staining for uidA expression. Exposure to At for 120
min proved more effective than shorter times, and elevated
concentrations gave more transformants than lower ones.   Long
exposure times and high concentrations, however, tended to reduce
shoot formation and elongation, with increased concentration having
the greatest effect.   Selection of putative transformants with K
proved workable, but even modest K concentrations inhibited
regeneration.   The selection process was therefore modified to
include culture on nonselective medium for 14 days before transfer
to selective medium.   This gave higher transformation frequencies
than otherwise obtained, without raising the frequencies of escapes
and/or partial or transient transformation.   The improvement is
thought to result from transformed calli forming and accumulating
meristematic centers sufficiently organized to survive the
selective antibiotic, withstand the effects of the many dying cells
surrounding them, and develop shoots for regeneration.

ATTACHMENT 2

Dinus, R.J., C.J. Stephens, and Shujun Chang. 1995. _Agrobacterium tumefaciens_-mediated transformation of eastern cottonwood (_Populus deltoides_). Abstr., p. 42, In: Program Book: International Poplar Symposium, August 20-25, 1995, University of Washington, Seattle, WA.

PLANNED ACTIVITY THROUGH FISCAL YEAR 1995-96

* Increase numbers of plants transgenic for GUS gene:

    Confirm transformation via biochemical/molecular assays

    Document permanence of gene expression

* Effect transformation of tobacco and model clone C175 with
    iaaM gene

* Regenerate plants transgenic for the iaaM gene; Repeat
    experimentation until plant numbers sufficient for
    assaying expression are available

* Confirm transformation via biochemical and molecular assays

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

FUTURE RESEARCH ACTIVITY

* Extend transformation protocol to other explant types,
    clones and/or species; emphasize elite plant materials of
    direct value to industry

* Characterize longer term effects of iaaM gene expression

RESEARCH PROPOSALS

IPST FOREST BIOLOGY
RESEARCH PROPOSALS
(Awarded, Submitted and in Review or Rejected)

Following is a list of 1994-1996 research proposals which have been submitted and awarded, rejected, or are currently under review. Following the list is a brief abstract or summary from each proposal submitted since our last PAC meeting. If you would like to see any of these proposals please contact one of the IPST authors.

Awarded Proposals (Approximately $221,000 Awarded To IPST in 1994-1996)

Title:	Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expression Studies.
Authors (Affiliation):	G. Pullman (IPST), J. Cairney (IPST), Jung H. Choi (GIT), Art Ragauskas (IPST)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$94,075 (IPST), $20,000 (GIT)
Period of Proposal:	Fiscal Year 1996 (July 1, 1995 - June 30, 1996)
Submitted:	13th July 1995
Status:	**Awarded, $75,000 to IPST, Fiscal Year 1996**

Title:	Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expression Studies (Matching Funds).
Authors (Affiliation):	G. Pullman (IPST), J. Cairney (IPST)
Awarding Agency:	Georgia Pacific Company
Amount Requested:	$ 10,000 ($10,000 to IPST)
Period of Proposal:	1996 (These Matching Funds are contingent on receipt of grant (same title) from Georgia Consortium in 1995.
Status:	**To be awarded in January, 1996 ($10 K to IPST Forest Biology)**

Title:	The Role of Peptide Processing in Plant Cell Growth and Development
Authors (Affiliation):	Ronald Dinus (IPST) and Sheldon W. May (GIT)
Awarding Agency:	Georgia Institute of Technology/Institute of Paper Science and Technology, Pulp and Paper Education Program
Amount Requested:	$ 38,990 ($ 19,681 to IPST)
Period of Proposal:	1995-1996
Submitted:	8th August, 1995
Status:	**Grant Awarded ($20 K to IPST Forest Biology, $20 K to GIT Dept. Chemistry & Biochemistry)**

Title:	Methods to Assay and Identify Populations of Bacteria Associated with Recycled Containerboard.
Authors (Affiliation):	Gerald Pullman
Awarding Agency:	Containerboard and Kraft Paper Group (CKPG)
Amount Requested:	$31,329
Period of Proposal:	1996-97
Submitted:	January 16, 1996
Status:	**To be awarded, $31,329 to IPST**

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Title: Identification and Potential Control of Bacteria Associated with Recycled Containerboard. Upon review, changed to Feasibility study for methods to assay and identify populations of bacteria associated with recycled containerboard.

Authors (Affiliation): Gerald Pullman (IPST), Ted Heindel (IPST), Alan Rudie (IPST). Proposal changed to one author, Gerald Pullman

Awarding Agency: Containerboard and Kraft Paper Group (CKPG)

Amount Requested: $62,000, changed to $6,000

Period of Proposal: 1995-96

Submitted: March 7, 1995

Status: **Grant awarded, $6,000 to IPST**

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 ($20 K to IPST Forest Biology, $20 K to GIT Biology)**

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 1993-1994 and 1994-1995 have already been granted. **Grant awarded for 1995-1996, $58,789 to IPST.**

Pending Proposals

Title: Molecular Methods for Induction of Early Flowering in Forest Trees.

Authors (Affiliation): Jeffrey F.D.Dean (UGA), Scott A. Merkle (UGA), Sarah Covert (UGA), Gerald Pullman (IPST) and John Cairney (IPST)

Awarding Agency: The Georgia Consortium for Technological Competitiveness in Pulp and Paper

Amount Requested: $230,000 ($120,000 to IPST)

Period of Proposal: 1997 Fiscal Year

Submitted: February 1, 1996

Status: In Review.

Title: Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expression Studies.

Authors (Affiliation): G. Pullman (IPST), J. Cairney (IPST), Jung H. Choi (GIT), Art Ragauskas (IPST)

Awarding Agency: The Georgia Consortium for Technological Competitiveness in Pulp and Paper

Amount Requested: $94,075 (IPST), $20,000 (GIT)

Period of Proposal: Fiscal Year 1997 (July 1, 1996 - June 30, 1997)

Submitted: 13th July 1995

Status: In Review (Ranked high for funding).

Title:	Genetically Engineering Sterility in Commercially Important Southern Trees
Authors (Affiliation):	John Cairney (IPST), Gerald Pullman (IPST), Ronald Dinus (IPST)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$68,000 (IPST)
Period of Proposal:	Fiscal Year 1997 (July 1, 1996 - June 30, 1997)
Submitted:	13th July 1995
Status:	In Review (Ranked high for funding)

Title:	Protecting the Fiber Supply Through Genetic Engineering of Drought Tolerance
Authors (Affiliation):	John Cairney (IPST), Gerald Pullman (IPST)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$68,000 (IPST)
Period of Proposal:	Fiscal Year 1997 (July 1, 1996 - June 30, 1997)
Submitted:	13th July 1995
Status:	In Review (Ranked high for funding)

Rejected Proposals

Title:	Tree Protection Through Biotechnology: Characterizing Proteinase Inhibitors in Transgenic Plants
Authors (Affiliation):	John Cairney (IPST), James C. Powers (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:	1995-1996
Submitted:	15 th July 1995
Status:	Not Funded

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:	Not Funded

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:	Not Funded

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: Not Funded

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: Not Funded

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: Not Funded

THE GEORGIA CONSORTIUM FOR
TECHNOLOGICAL COMPETITIVENESS IN PULP AND PAPER

FIBER SUPPLY DIVISION
FY 97 PROJECT APPLICATION

1. **TITLE:** *Molecular Methods for Induction of Early Flowering in Forest Trees*

2. **INVESTIGATORS:**

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3. EXECUTIVE SUMMARY

Progress in the genetic improvement of forest trees has traditionally lagged behind that in agronomic species due to the long life cycles and large sizes of forest trees. Not only have these problems dictated the pace of tree improvement for the leading commercial forest species, but they have also limited genetic improvement programs to a handful of North American trees. Tree breeding could be greatly accelerated if trees could be induced to flower while they are still seedlings--that is, within 1 or 2 years following germination. Not only would this dramatically shorten the breeding cycle, but it would also make breeding in the controlled environment of the greenhouse possible. The ability to induce accelerated flowering in forest trees would also provide a critical tool for a related project in which we intend to test strategies for the production of sterile trees (proposal submitted to GCTCPP for FY 1997). Finally, induction of early flowering in forest trees would enable us and other researchers to rapidly characterize the inheritance of inserted DNA in the progeny of transgenic trees, something which has yet to be accomplished, even though such trees have existed since 1989.

Recently, research with a group of flower-meristem-identity genes in the herbaceous model plant, Arabidopsis, has culminated in the production of transgenic Arabidopsis and hybrid aspen plants in which precocious flower development was induced. In one study, hybrid aspen plants transformed with the Arabidospsis LEAFY gene (LFY) under the control of the cauliflower mosaic virus 35S promoter produced flowers when they were only 5 months old. We propose to demonstrate that the strategy used to induce precocious flowering in transgenic Arabidopsis and hybrid aspen (i.e. fusion of a constitutive promoter to genes encoding flower-meristem-identity genes) can be applied to induce precocious flowering in commercially important southeastern hardwoods (yellow-poplar, sweetgum, and cottonwood) and precocious strobilus production in conifers (loblolly pine). Future objectives include transforming these same species with genetic constructs in which inducible promoters have been fused to the Arabidopsis flower-meristem-identity genes, as well as cloning and characterizing one or more flower-meristem-identity genes from tissues of the tree species under study.

The ultimate goal of this research is to generate trees in which the timing of flower production can be controlled, enabling accelerated breeding for operational tree improvement and accelerated testing of sterility strategies. In projects previously supported by the GCTCPP, we have developed the expertise necessary to complete the work proposed here; that is, we have produced transgenic trees expressing foreign genes and have rapidly cloned tree genes using DNA sequences conserved in other organisms.

This proposal represents a new collaboration between researchers at the University of Georgia and the Institute for Paper Science and Technology. A significant component of this collaboration will be to bring together the substantial skills that IPST researchers have demonstrated in somatic embryogenesis and molecular biology of loblolly pine with the expertise demonstrated by UGA researchers in using microparticle bombardment to generate transgenic plants. By performing this work in a collaborative effort, we can assure that techniques for inducing controlled precocious flowering will be developed as rapidly as possible for the premier commercial tree species of the Southeastern U.S. (loblolly pine). The program described in this proposal will also be tightly integrated with projects to develop techniques for production of sterile hardwood and softwood trees as described in proposals previously submitted by UGA and IPST researchers for consideration by the GCTCPP.

Precocious flowering and sterility represent opposite sides of the same coin, yet they both hold enormous potential value for the forest products industry. Genetic engineering provides the means for rapidly introducing valuable new traits into trees. Controlled precocious flowering would allow us to fix those new traits in the genetic background or move them into new backgrounds through greatly speeded breeding programs. These accelerated breeding programs should enable us to bring loblolly pine breeding on a par with the *Eucalyptus* breeding programs that have led to such rapid improvements in that species over the past two decades. However, we will be prevented from planting genetically engineered trees widely in the environment if we cannot prevent their outcrossing to surrounding populations of wild, native trees. Thus, the simultaneous pursuit of techniques for inducing precocious flowering and forced sterility through a set of integrated and collaborative programs put forth by UGA and IPST researchers provides the best hope for the rapid development of tools that will be critical for southeastern forestry in the next century.

Keywords: tree improvement, genetic engineering, precocious flowering, sterility

2

Project 4107 Continuation

Project 4107-95 -- Methods to Assay and Identify Populations of Bacteria Associated with Recycled Containerboard

Jerry Pullman, Shannon Johnson

Executive Summary: The Containerboard Industry is interested in monitoring bacterial populations in containerboard produced from virgin and recycled fiber in support of continuing to provide customers with a high quality, safe product. Methods of assaying bacterial populations of importance to the Containerboard Industry were presented in Project 4107-95. These methods need to be verified and proven acceptable to the Industry. With reliable methods in place, a multiple mill survey of the bacteria found in containerboard product can be conducted. Evaluations can then be made by the Industry or individual mills whether research on non-chemical bacterial control strategies is desired.

Research Proposed for 1996

I. Objectives / Goals:

1) Verify membrane filtration test for total coliforms and *E. coli* as a suitable standard method for industry. Confirm with TAPPI, industry, and appropriate experts. This method was developed during Project 4107-95-- Feasibility Study for Methods to Assay and Identify Populations of Bacteria Associated with Recycled Containerboard.

2) Survey bacterial populations in linerboard produced with none, part or all recycled fiber. Assay product from approximately 20 CKPG member mills varying in location, product, and process.

3) Report findings to CKPG. Propose next steps.

II. Estimated Time

The work proposed will be completed during 1996. This work is part of a multi-phased program intended to provide additional methods to monitor important bacterial populations in virgin and recycled linerboard and survey a portion of the industry for linerboard bacterial content. If bacterial populations identified are of concern to the industry, future phases of this research will determine bacterial population changes through the linerboard-making process, and provide and evaluate non-chemical control strategies.

III. Estimated Cost - Total for 1996/1997 (1 year)

Salaries:

Senior Professional		
Jerry Pullman (0.02)		$ 1,500
Senior Technician (0.10 time)		4,830
Technician III (0.10 time)		3,150
Total Direct Salaries		$ 9,480
Fringe Benefits (22%)		2,086
Total Salaries and Benefits		$ 11,566
Out-of-Pocket Expenses		
Outside Services (Consultation with experts)		2,000
Supplies & Materials		2,500
Library Costs		150
Total Out-of-Pocket Expenses		4,650
Total Direct Charges		16,216
Indirect Costs (68%)		11,027
Service Fee (15%)		4,086
Total Proposed Budget		$ 31,329

IV. Scope

The increased use of recycled materials in the containerboard industry may contribute to increasing amounts of bacteria in the linerboard product. This may become a significant issue where recycled materials come in contact with food products. With reliable methods to identify classes of important bacteria and an understanding of bacterial population changes during the recycling process, an evaluation can be made if a potential problem exists and some potential control strategies can be proposed.

V. Value to Industry

There is a need to identify the type and count of bacteria present in recycled containerboard. If bacterial problems persist, it is likely that recycled materials will see increased regulation. In addition, the customer may turn elsewhere for substitute products or to companies with microbial quality control programs. If significant populations of bacteria are found, a future question would concern populations after repeated recycling. AF&PA reports that in 1993, nearly 16.7 million tons of old corrugated material were recovered in the United States for recycling, representing more than 62% of all the containerboard used. Approximately 58% of the recovered containerboard goes back into new containerboard. Therefore, repeated bacterial contamination could become a problem depending on how the end-product is used.

VI. Approach

Establish credibility of methods identified during Project 4107-95. Verify methods with appropriate experts. Survey additional mills (approximately 20) across the virgin and recycled linerboard industry.

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., all requirements completed, will matriculate in June, 1996)
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 by 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.) * Completed M.S. Degree June, 1995
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.)   Completed M.S. Degree June, 1995
Title: Lignin and Holo-Cellulose Contents of *Pinus taeda* Progenies using a FTIR Analytical Technique.
Advisor: Ron Dinus

Summary: The Lignin content, specific gravity, tree height, and DBH of 123 *Pinus taeda trees*, each 35 years old, representing 20 half-sib families, was measured. The wood samples were taken from the trees by 12 mm increment cores. The lignin content of increment core samples was estimated using an FTIR technique. The specific gravity of cores was measured using the maximum moisture content method. The tree DBH and height were measured in the field using a hypsometer and diameter tape.

The samples were prepared for FTIR by oven drying, extracting with DCM, and grinding to pass a 80 mesh screen. Approximately 100 mg of each sample was scanned using a diffuse reflectance cell. Derivative minimization was employed to determine the amount of lignin present in each sample. The method was calibrated using known mixtures of holocellulose and 1,4 dioxane extracted lignin. The results obtained were compared with sodium chlorite holocellulose content data.

The narrow-sense heritability for individuals and families was calculated for each characteristic measured. The heritability and range data were used to estimate genetic gains for individual and family selection. Correlations between wood volume, specific gravity, and lignin content were examined for families and individuals, no correlations were found.

The range in lignin contents for individuals and families was found to be from 16.4 to 31.4% and 23.9 to 29.1% respectively. The lignin content heritability was found to be 0.11 for individuals and 0.77 for families. These values estimated that a genetic reduction of 2.0% for family selection and 0.9% for individual selection should be possible based on selecting the lowest 5%.

Relevant literature in the fields of tree improvement and FTIR techniques was reviewed. The results from other work were compared in methodology and results with this research.

Dana Freeman (M. Sc.) *

Title: The Role of Peptide Processing in Plant Cell Growth and Development.

Advisor: Jerry Pullman and John Cairney

Summary: Bioactive peptides are involved in practically every cellular function in animal cells, but in the cells of plants, it is just now becoming apparent that peptides play a part in cell growth and development. Bioactive peptides are generated from precursors through post-translational modifications, in particular, carboxy-terminal amidation. The amide group is required for bioactivity and is speculated to play a role in receptor binding and regulation. Two "amidating enzymes", Peptidylglycine a-Monooxygenase (PAM), and Peptidylamidoglycolate Lyase (PGL), have been demonstrated to function in carboxy-terminal amidation of glycine-extended substrates. A number of substrates, inhibitors, and assays have been developed to detect the presence of PAM and PGL enzymes.

A recent discovery of a 37 amino acid peptide isolated from soybean was found to stimulate the phosphorylation activity of a soybean receptor protein, suggesting a role for the peptide in signal transduction. The peptide has a glycine at its C-terminus, and via mass spectral evidence it is revealed that a portion of the peptide is processed to delete this terminal glycine. Through this evidence, it is highly suggestive that amidative processing of bioactive peptides may occur in plant cells.

The objective of this research is to explore whether amidative processing of bioactive peptides occurs in plant cells of both angiosperms and gymnosperms. The research will be carried out with Eastern cottonwood (*Populus deltoides*) and Loblolly pine (*Pinus taeda*). Research with cottonwood seems ideal because the first finding of bioactive peptides in plants was reported for soybean, another dicotyledonous Angiosperm. Loblolly pine embryos exhibit several measurable variations (cell elongation, cell wall development, cell differentiation) that may possibly be altered in the presence of amidation inhibitors. The goal of the research is to obtain evidence for bioactive processing and to determine what consequences inhibition of such processing may have on plant cell development and growth. The experimental approach will be to make use of the selective and potent amidation inactivators and inhibitors by determining their effects on plant cells in culture.

Jeff Grass (M. Sc.) *

Title: Molecular Characterization of Floral Homeotic Genes Expressed in *Pinus taeda* and *Populus deltoides*.

Advisor: John Cairney

Summary: The pulp and paper industry supports research in genetic engineering with the goal of developing an improved source of raw material. Traditional breeding techniques have been used to successfully achieved this goal in the past. However, the techniques developed by genetic engineering are proving to be more economical than traditional breeding techniques. State and federal agencies strictly regulate the release and use of genetically engineered organisms in the environment. The greatest concern of the agencies is the transfer of selected genes from one organism. It is hoped that sterile organisms will express lower than normal rates of gene transfer.

The goal of the project is to identify regulatory genes expressed in reproductive tissue of loblolly pine and cottonwood trees. These genes will be used to genetically engineer sterile trees for use in the pulp and paper industry. Sterile organisms can be genetically engineered by disrupting the function of regulatory and structural genes responsible for the development of reproductive tissue. Introduction of modified copies of these genes can cause selective tissue death. The desired genes will be identified using information from previously discovered genes with similar known functions. Once the desired genes have been identified, they will be sequenced and isolated.

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.) *   Completed M.S. Degree June, 1995
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.

Eric Wagner (M. Sc.) *
Title: Using the NMR in Plant Morphology and Embryo Development
Advisor: Jerry Pullman and Art Ragauskas
Summary: I plan to investigate *Pinus taeda* (loblolly pine), a tree species important to the pulp and paper industry, using Nuclear Magnetic Resonance (NMR) to gain knowledge about how plant embryos mature. Despite its limited use in this area, Nuclear Magnetic Resonance Imaging has proven itself as an extremely useful tool in medicine due to its relatively benign effects on living tissues. Emphasis will be placed on mapping intracellular pH, but nuclear resonance can also provide important information about energy utilization and other metabolic functions in the cell. Depending on time constraints, I will investigate other physiological aspects of the somatic embryos deemed consequential.

Byron Waldrop (M. Sc.) *
Title: Assessing Somatic Embryo Quality Using Differential Display Techniques
Advisor: John Cairney
Summary: Somatic embryogenesis offers forest geneticists and planters opportunities to multiply superior plants, multiply clones of genetically altered plants, and store genetic information for future plantings. This technique promises to be a powerful tool for insuring an adequate supply of high quality raw material for the paper industry's future. However, the adaptation of somatic embryogenesis techniques to commercially valuable species, such as conifers, has been problematic. It has been difficult to identify high

quality embryos, and rates of induction and maturation remain very low compared with those of non-woody plants. Analysis and comparison of gene expression in zygotic and somatic embryos are the keys to understanding these problems. If a gene, or series of genes, are found to be expressed by only a certain quality embryo or only during a certain stage of development, these genes can be used as markers for the classification of other embryos. Comparison of genes that are expressed by zygotic embryos but not by somatic embryo at the analogous developmental stage, may reveal nutritional or environmental imbalances present in the somatic embryogenesis protocol, thus allowing for corrections to be made. The proposed study will use differential display techniques to compare gene expression during developmental stages of zygotic and somatic embryos. Genes whose expression is found to be specific to a growth condition or developmental stage will be investigated further by isolating, cloning, and sequencing cDNA fragments from gels. The information gathered will then be used to identify high quality embryos, and guide protocol adjustments directed at improving somatic embryo quality.

Vincent Ciavatta (Ph. D.) *
Title: To be determined.
Advisor: John Cairney
Summary: Vinny recently entered the Ph. D. program at IPST and is currently working on the A390, A490 problem-solving sequence. A Ph. D. project within the Forest Biology Molecular Biology area will be developed over the next year.

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: Data from the pilot scale trial at Anditz Sprout-Bauer has been analyzed. Strength increases evident on the laboratory scale were consistent in the pilot scale work, but pilot scale results were less pronounced. This study has shown that Cartapip treatment of wood chips for TMP processing yields pulps with lower extractives contents and increased strength properties. Tear strength increased as much as 89% over non-treated, non-aged controls. A dissertation draft is currently under review. The work will be defended within the next month. Papers submitted to the International Conference on Biotechnology in the Pulp and Paper Industry and the TAPPI Pulping Conference have been reviewed and accepted for publication.

Rene Kapik * (Ph. D. Completed June, 1995)
Title: A study of plant growth regulators during zygotic embryogenesis in loblolly pine.
Advisor: Ron Dinus
Summary: Dissertation is available upon request.

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 activated carbons supplied by Sigma revealed that one carbon promoted embryogenesis of Douglas-fir while the other did not. The goal of this project is to discover why one carbon was ineffective. Research will be directed towards physically and chemically characterizing many (~20+) different carbons with the goal of correlating these characteristics with carbon performance in tissue culture medium. Activated carbon 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 results indicate that the two carbons have different porosity, different surface charge, and different ionic content. A liquid Norway Spruce initiation system has been successfully demonstrated for use as a bioassay.

Colleen Walker (Ph. D. Completed 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: Dissertation available upon request. (A summary of this research was published in the June edition of TAPPI Journal.

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. A literature review based on this dissertation research proposal was published in the February issue of Progress in Paper Recycling.

PUBLICATIONS

PUBLICATIONS - 1994-1996
(Issued, in press, or submitted)

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