

ANNUAL PROGRAM REVIEW

FOREST BIOLOGY

March 26-27, 1998

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SOFTWOODS

**DUES FUNDED RESEARCH CONSORTIUM
1997-1998**

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

Status Report for Project F010

**Gerald Pullman
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Heidi Schindler
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March 26-27, 1998

**DUES-FUNDED PROJECT SUMMARY
FY 1997-98**

Project Title: MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

Project Code: SFTWD

Project Number: F-010

PAC: FOREST BIOLOGY

Division: CBS

Project Staff

Faculty/Senior Staff: Jerry Pullman, John Cairney, Gary Peter, John MacKay

Staff: Barbara Johns, Paul Montello, Xiaorong Feng, Theresa Vales, Cielo Castillo, Yalin Zhang, temporary help

FY 97-98 Budget: \$460,826

Allocated as Matching Funds: \$ 46,804 (10.2%)

Time Allocation

Faculty/Senior Staff: 1.1

Support: 4.0

Supporting Research

M.S. Students: 3

Ph.D. Students: 2

External: 1997 \$504,037, Nanfei Xu, Ranjan Perera, Lin Ge

RESEARCH LINE/ROADMAP: Improve the fiber productivity of North American lands so that they are competitive in the world pulpwood market

PROJECT OBJECTIVE: Develop reliable cell & tissue culture systems for the mass clonal propagation of genetically improved softwoods.

PROJECT BACKGROUND:

A continued supply of low cost, high quality raw materials is essential for the future success of the U.S. forest products industry. The continual loss of forest lands to urban growth, continued and new environmental regulations, and the rapid growth occurring in competitive forest plantations abroad put intense pressure on U.S. companies to increase the yield of wood per acre. If the industry is to grow, it must sustain reliable low cost sources of raw materials. The clonal propagation of high value forest trees from breeding and genetic engineering programs has the potential to help meet future industry needs by increasing forest yields and improving raw material uniformity and quality.

SUMMARY OF RESULTS: (Brackets show support from student research or outside projects.)

Faculty / Senior Staff Forest Biology Team is in place. Dr. John MacKay joined the team this past summer. Team is skill based and focused on multiplication of high value trees through somatic embryogenesis.

External funding for research related to both F-010 and F-011 increased to approximately \$504, 000.

The Institute's first crop of loblolly pine somatic seedlings did well during the past summer. Thirty-five trees, produced from a single clone of somatic embryos, were planted last winter. All of the trees survived transfer to the field and grew approximately 1 foot during the past growing season.

Cryogenic storage of loblolly pine somatic embryo cultures in liquid nitrogen is working well and has become routine. Improved protocol has been developed which saves time, labor, materials and shows improved survival rates.

Comparisons of germinating somatic and natural seed embryos confirm that our somatic embryo functions normally but is immature. Current somatic embryos behave similar to zygotic embryos that have proceeded through approximately ½ of their development cycle.

Culture cycling has been studied and a pH cycle has been observed; pH peaks and troughs precede volume peaks and troughs by one week. Bands (cDNAs) which correspond to pH peaks and troughs have been isolated and are being analyzed. (Student research)

Ph.D. student research has shown that activated carbon significantly alters the final pH of tissue culture medium. Changes in pH in turn alter the availability of specific ions resulting in unwanted ion excess or deficiency. (Student research)

A new approach has been taken to improve somatic embryo quality. Tissue culture media will be formulated by a combination of metal analysis during natural embryo development and the comparison of metals found in natural and somatic embryos at different times. This approach is already yielding beneficial results.

- Metal analyses of full term seed indicate that metal targets are similar regardless of tree location or genetics.
- Analyses of embryo and surrounding tissues over the developmental sequence show sequence patterns.
- Analyses of somatic embryos show excess or deficiencies in ion content.
- Maturation media changes based on the above elemental analyses have already produced statistically significant improvements in embryo yield.
- Maintenance media changes based on the above elemental analyses are producing statistically significant improvements in embryo yield.

Embryo quality improvement has been achieved by adjusting the abscisic acid amount and sequence combined with increasing the time on maturation medium.

Investigations into two important environmental factors during embryo development have resulted in statistically significant increases in embryo yields. By improving gas exchange through a change in the plate wrapping material and increasing the nutritional reserves available by changing to a larger plate and medium amount have both resulted in statistically significant increases in embryo yield.

First estimates were obtained for the percent of early-stage embryos that mature into cotyledonary embryos.

Methods have been developed to implement automated counting of cotyledonary embryos.

The molecular biology based differential display technique is working well in the laboratory. Results are showing repeatable and comparable banding patterns for developmentally staged natural and somatic embryos of loblolly pine. Differences and similarities in gene expression between natural and somatic embryos are being observed for the first time. IPST is becoming a world leader in adapting and applying this technology to plant embryogenesis.

- Differential display has been applied successfully to single late stage loblolly pine embryos.
- Over 400 cDNAs from mRNA expressed at different staged embryos have been identified & cloned (4130).
- 300 of these genes have been sequenced (4130).
- A method (Dot Array Assays) has been developed to simultaneously follow the activity of hundreds of genes in developing embryos (4130).
- Isolated cDNAs which are expressed at specific stages of development. These can act as markers to follow embryo development in the lab. (4130)

Experiments have been initiated to study the role of the suspensor in loblolly pine embryos. The suspensor is thought to play an important role in embryo nutrition and regulation of embryo growth and development. Experiments to identify genes active in the suspensor are ongoing and have used a method called

subtractive hybridization. Already preliminary information indicates the importance of suspensor-formed storage proteins, likely involved in nutrition during early embryo development.

- Over 350 high quality cDNAs representing genes active in the suspensor have been cloned and analyzed.
- Several of these cDNAs were confirmed to be specific or more abundant in the suspensor of early stage embryos
- Sequencing of only a few cDNAs has already identified genes for three classes of seed storage proteins

A Loblolly Pine seed storage protein cDNA has been cloned and its expression followed through embryo development. This clone provides a valuable marker gene to follow late embryo development in the laboratory (4187).

Ph.D. student research has isolated cDNAs expressed at early stages of development. These can act as markers to follow early embryo development. Note at this point anatomical features may be small and difficult to see. (Student research)

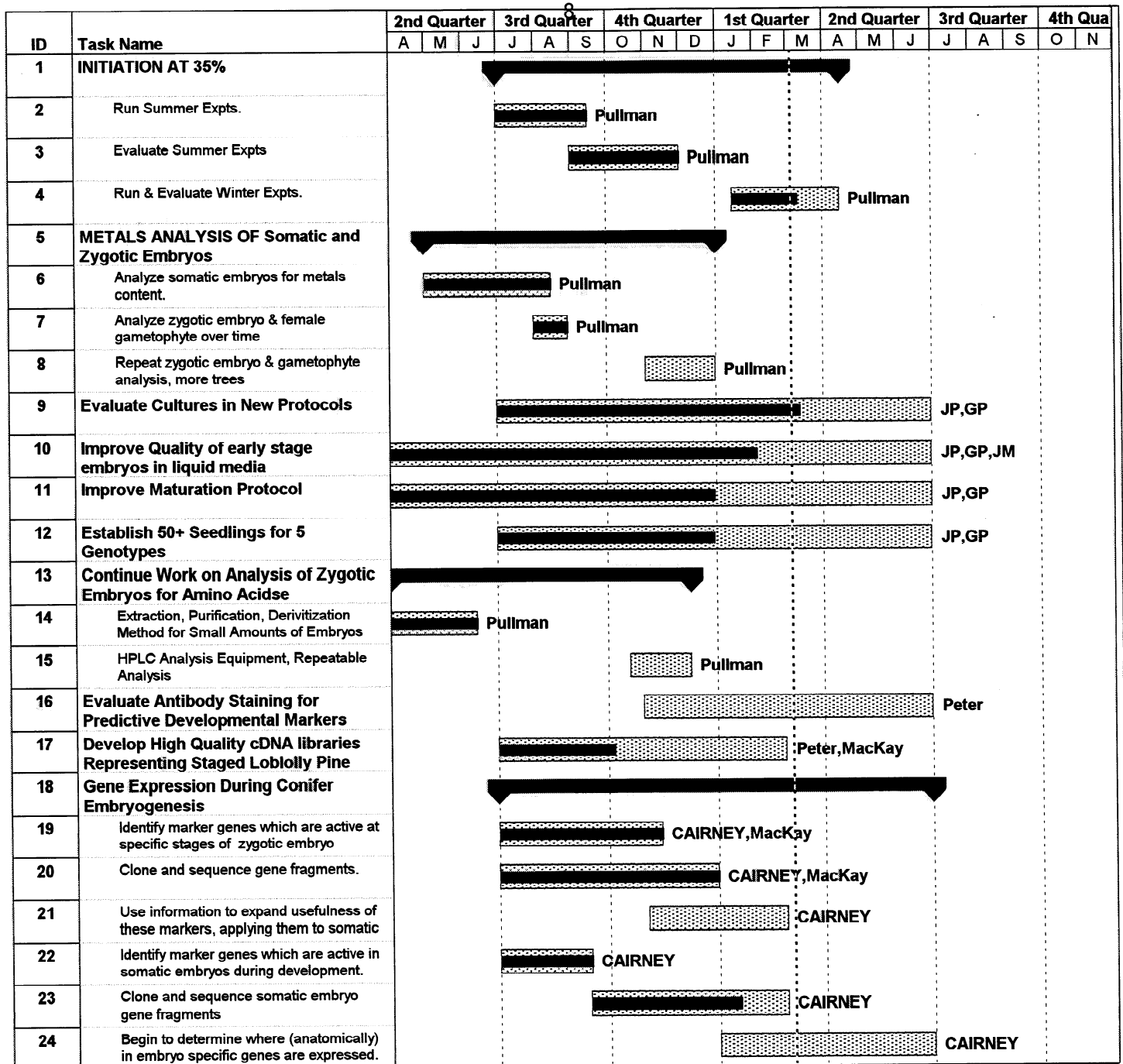
GOALS FOR FY 97-98:

1. Improve initiation protocol to meet target of 35%.
2. Metals analysis of somatic and zygotic embryos.
 - Analyze somatic embryos for metals content.
 - Analyze female gametophyte tissue over embryo development cycle.
3. Evaluate new cultures for performance in current Protocols.
4. Improve quality of early-stage embryos in liquid media.
5. Improve maturation protocol .
6. Establish 50+ seedlings from each of 5 genotypes.
7. Continue work on analysis of zygotic embryos for amino acids.
8. Evaluate concept of antibody staining for predictive developmental markers.
9. Develop high quality cDNA libraries representing staged loblolly pine zygotic embryo development.
10. Gene expression during conifer embryogenesis.
 - Identify marker genes which are active at specific stages of zygotic embryo development.
 - Clone and sequence gene fragments.
 - Use information to expand usefulness of these markers, applying them to somatic genotypes.
 - Repeat steps above for somatic embryos.
 - Begin to determine where (anatomically) in embryo specific genes are expressed.

DELIVERABLES:

Report describing results and research progress in the fall of 1997.
Annual report describing results and research progress in the spring 1998.

SCHEDULE (See Attached Timeline):



Project: F010 Goals Gant Chart Date: Thu 3/5/98	Task		Rolled Up Task	
	Progress		Rolled Up Milestone	
	Milestone		Rolled Up Progress	
	Summary			

**MASS CLONAL PROPAGATION OF IMPROVED CONIFERS -
INITIATION & CULTURE SURVIVAL**

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Summary

Results from Summer 1997 initiations continue to confirm earlier initiation findings. The average initiation rates per ½ sib family on medium 505 during summer 1997 ranged from 0-26.7% with an average of 8.5%. This compares well with the overall medium 505 average 1996 initiation rate of 7.4%. During the Summer 1997 initiation trials approximately 10,000 explants were placed on medium resulting in 752 initiations for an overall all media 7.6% rate, compared to 5.7% rate in 1996. Our control medium currently is a combination of 1/2 P6 Salts, activated carbon at 37.5-50 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 2 g/l, and hormones consisting of 2 ppm NAA, 0.45 ppm BAP and 0.43 ppm Kinetin. The development of this medium has been covered in previous PAC reports.

During our tests of many improvement hypotheses two factors stand out as repeatable and statistically significant improvements. The addition of abscisic acid or silver nitrate to initiation media significantly improved initiation rates and the combination of both of these ingredients gave the highest initiation rates. While these improvements move us closer to our target of 35% initiation, other laboratories also apparently experimented with the addition of abscisic acid to initiation medium over the past several years resulting in patent applications.

As requested by PAC we have carefully examined two factors for effects on initiation. Both time in cold storage and average embryo stage (between 1-4) were thought to impact initiation percent. However, data for the 27 mother trees used during 1996 and 1997 show little correlation between embryo stage or days in cold storage and percent initiation. It is clear that much of the trial to trial variation with the same explant cone material remains to be explained.

Improved survival of initiated cultures remains a research focus point. Loss of nearly 3/4 of the initiated cultures represents a major challenge for commercialization of this technology. Recent experimentation with early transfer of initiated cultures to liquid medium increased culture survival to 42% when 38 of the 1997 summer initiations were recently tested.

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 that we report are for successful progression through these three steps resulting in at least three visible somatic embryos emanating from a zygotic embryo(s).

1997 Overall Initiation Frequencies and Comparison with Past Years

Table 1 shows a summary of initiations for all media and all 1/2 sib families used during Summer 1997 initiation experiments. Cones were collected from eighteen families. All families contained early zygotic embryos at stages 2-4 that were suitable for experimentation. Fourteen of the eighteen families were able to initiate cultures with initiation rates ranging up to 47.7%. Table 1 also compares initiation rates for medium 505 during 1995, 1996, and 1997 for 32 loblolly pine mother trees. Overall 8.5% initiation occurred across all families during 1997.

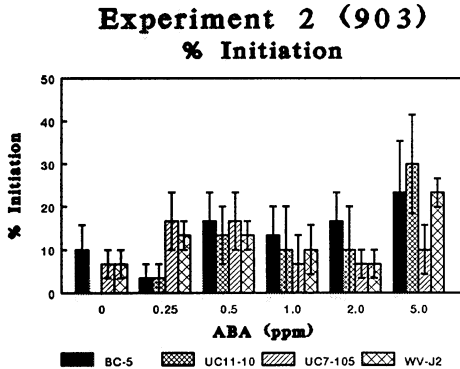
Initiation Improvements

Our program continually strives to reach our target of 35% loblolly pine initiation among many families. In Summer 1995 we began investigating the hypothesis that addition of abscisic acid to the initiation medium would improve initiation rates. This hypothesis was based on the fact that Renee Kapik's doctoral thesis showed significant levels of ABA present during the period of early embryo development when natural cleavage polyembryony (multiplication) occurs. This suggested that embryo initiation and maintenance may be improved by the addition of ABA. Over the past two years this hypothesis has been confirmed and optimized to a point where the initiation medium has been improved. Other laboratories also apparently experimented with the addition of abscisic acid to initiation medium over the past several years. Patent # 5,677,185 was recently granted to Westvaco Corporation for the use of ABA during somatic embryo initiation for a list of *Pinus* species. New Zealand researchers (Jenny Aitken-Christie of Baker Holding Company) also applied, a few days after Westvaco, for a world patent on a similar concept for initiation in conifers or other woody species. We will attempt to document the key IPST experiments that lead to this improvement in our initiation system. Unless otherwise mentioned all experiments are modifications as described of medium 505. Table 2 shows the composition of medium 505.

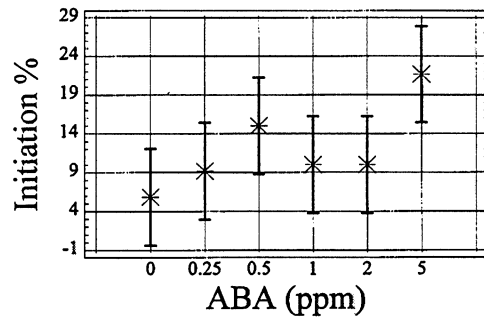
Experiment 1 (889). An early version of medium 505, containing 1 g/l of Gelrite instead of 2 g/l, was tested for extrusion and initiation as a control or with the addition of 1 ppm ABA. Among three mother trees tested with 30 explants each, the control medium achieved 28% extrusion and 4.5% initiation. The test medium, with added 1 ppm ABA, achieved 29% extrusion and 9% initiation. A additional pair of media both had the 50 mg/l of activated carbon removed; these achieved 25% extrusion and 1% initiation without ABA vs. 25% extrusion and 9% initiation for

medium with 1 ppm ABA. While these differences were not statistically significant they suggested further experimentation.

Experiment 2 (903). Medium 505 was used as a control and ABA was added to medium 505 in amounts of 0, 0.25, 0.5, 1.0, 2.0, and 5.0 ppm. Results showed increased extrusion and initiation with all levels of added ABA.

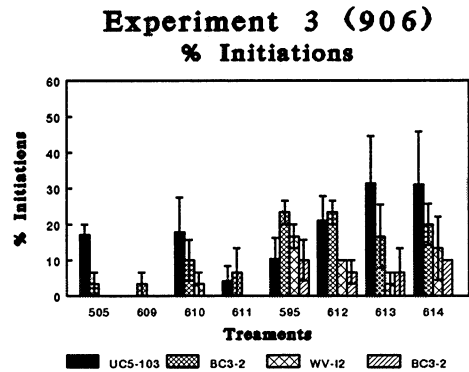


Means & 95% Confidence Intervals Expt. 903

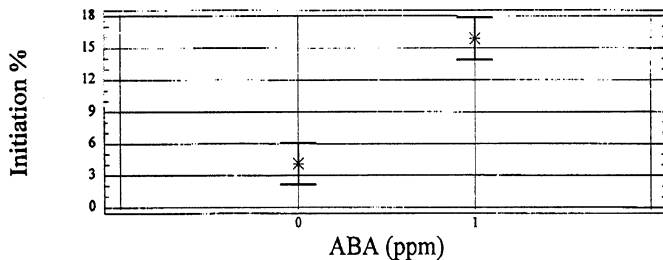


Experiment 3 (906). Medium 505 was tested in a 2 x 4 factorial arrangement with two levels of ABA (0, and 1 ppm) and four levels of glutamine (450, 650, 1000, and 1250 ppm). Results indicate that initiation was significantly increased by adding 1 ppm ABA to medium 505. Initiation was not significantly different with changing glutamine levels.

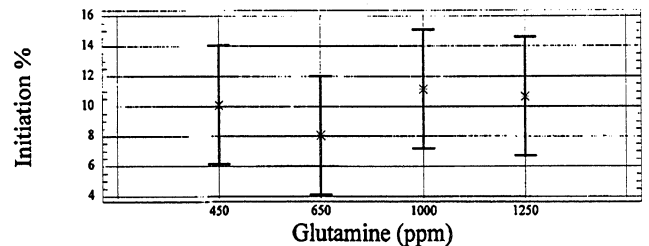
Media	ABA	Glutamine
505	0 ppm	450 ppm
609	0	650
610	0	1000
611	0	1250
612	1	450
613	1	650
614	1	1000
615	1	1250



95% Confidence Intervals Expt 906



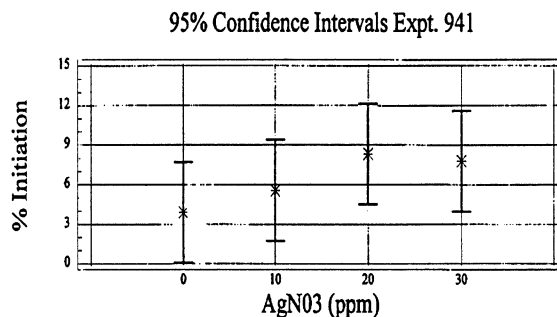
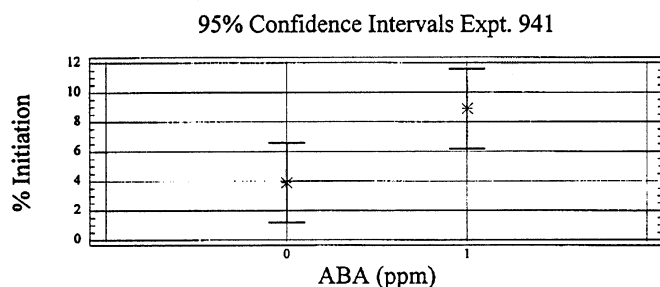
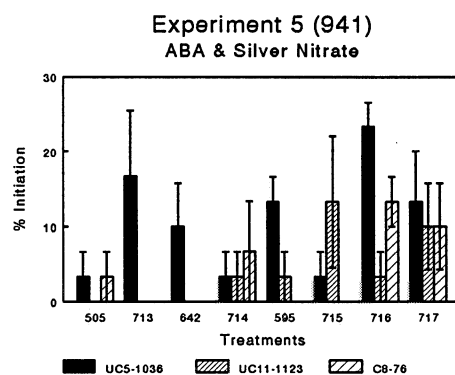
95% Confidence Intervals for Expt 906



Experiment 4 (910). Medium 505 was tested alone or supplemented with several ethylene inhibitors. Nickel chloride at 20 μM (medium 640) and silver nitrate at 20 μM (medium 642) showed increased initiation rates; with differences between 505 and 642 being statistically significant.

Experiment 5 (941). During Summer 1997 medium 505 was tested in a 2 x 4 factorial arrangement. Two levels of ABA (0 and 1 ppm) were combined in all combinations with 4 levels of silver nitrate (0, 10, 20, or 30 μM). Again medium with ABA showed significantly higher initiation rates. The combination of ABA and silver nitrate further increased initiation. The amounts of 20-30 μM silver nitrate appeared optimal.

Media	ABA	AgNO ₃
505	0 ppm	0 μM
713	0	10
642	0	20
714	0	30
595	1	0
715	1	10
716	1	20
717	1	30



Two additional experiments were carried out combining medium 505 with 1 ppm of ABA alone, 10-30 μM silver nitrate alone, or these two materials together. All experiments showed improved initiation rates with the addition of ABA alone. Silver nitrate alone was not beneficial. A combination of ABA and silver nitrate often caused the highest initiation rates. The most consistently beneficial amount of silver nitrate combined with ABA was 20 μM .

Effects of Embryo Stage and Cone Cold Storage on Initiation

Over the past year PAC has recommended that we re-examine our initiation data to determine if initiation has actually decreased from prior years' results. As one can see from Table 1, initiation percentages vary greatly with mother tree cone source. However, it does appear that some trees consistently provide high initiation rates while others consistently show little initiation. Yearly data will vary with the combination of mother trees used. Since our program strives to develop systems that work across many genotypes, it is to our advantage to vary some mother trees from year to year.

With the availability of several years of initiation data on medium 505 we were at last able to carefully analyze several factors for their effects on initiation. Both time in cold storage and average embryo stage (between 1-4) were thought to impact initiation percent. However, data for the 27 mother trees used during 1996 and 1997 show little correlation between embryo stage or days in cold storage and percent initiation (Figures 1a and 1d). Additional comparisons plotting embryo stage or days in cold storage against the initiation for specific cone mother trees also shows no trend even for individual trees (Figures 1b, 1c, 1e, 1f). It is clear from our trials that much unexplained variation occurs from trial to trial with the same explant cone material. Additional hypotheses will be investigated this year to account for this variation.

Figure1. Percent initiation for loblolly pine on medium 505 during 1996-1997. a) Effect of embryo stage on initiation rate for 27 tree cone sources over two years. b) Effect of embryo stage on initiation rates for specific cone source mother trees BC-5, BC-9, UC11-1055, UC11-1057, UC11-1069, and UC7-1051 over two years. c) Effect of embryo stage on initiation rate for specific cone source mother trees WV-H, WV-I, WV-J, and WV-F. d) Effect of cold storage on initiation rates for 27 tree cone sources over two years. e) Effect of days in cold storage at 4-5C on initiation rates for specific cone source mother trees BC-5, BC-9, UC11-1055, UC11-1057, UC11-1069, and UC7-1051 over two years. f) Effect of days in cold storage at 4-5C on initiation rates for specific cone source mother trees WV-H, WV-I, WV-J, and WV-F.

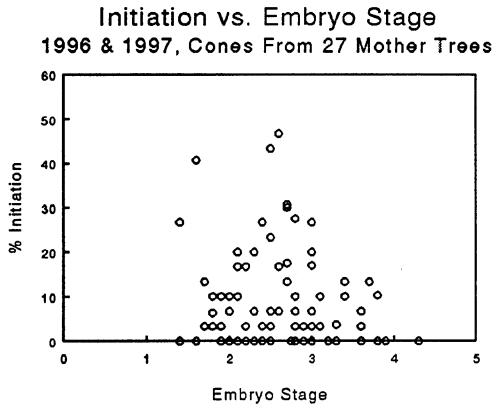


Figure 1a

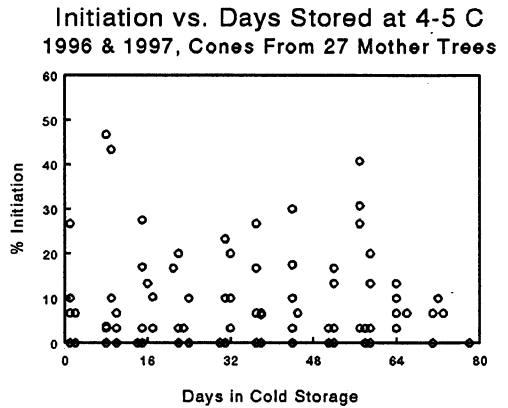


Figure 1d

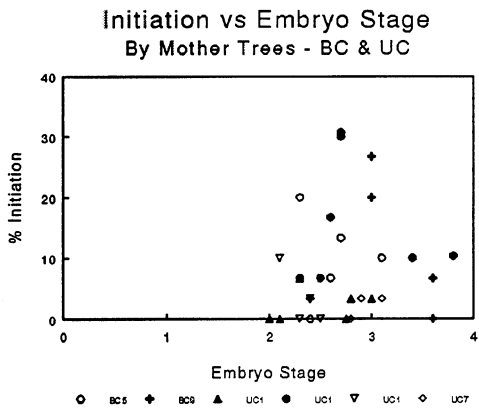


Figure 1b

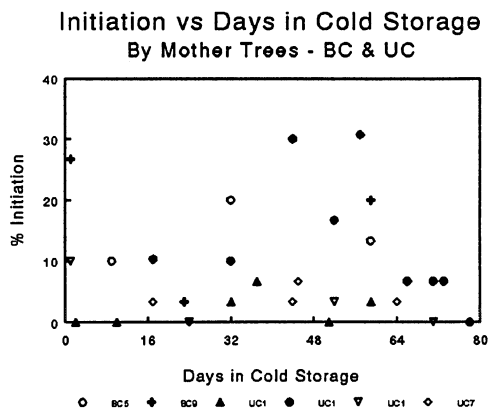


Figure 1e

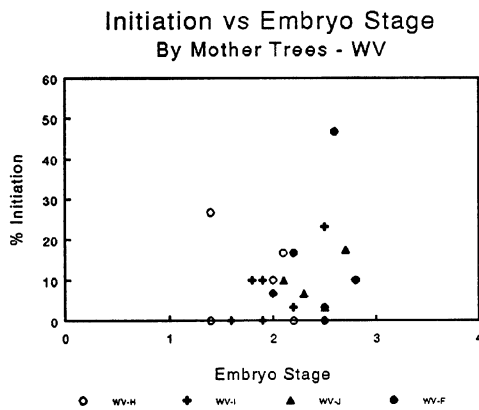


Figure 1c

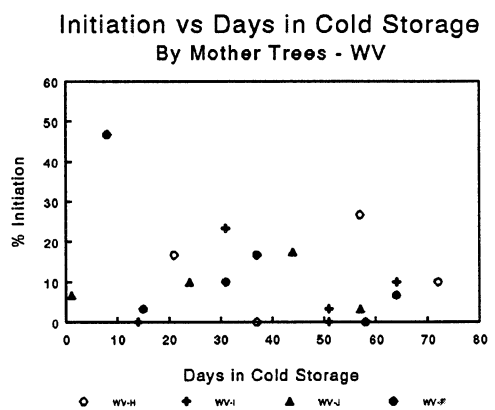


Figure 1f

Culture Survival

With an increased number of successful initiations in the past several years we have been able to better evaluate long-term culture survival. Two tests with large numbers of new initiations have shown survival as follows: 22% survival of 436 of the 1995 initiations (PAC report Spring 1996), and 33% survival of 765 of the 1996 initiations (PAC report Spring 1997). Significant improvement in culture maintenance is necessary. Culture survival of 50% is now one of our focus areas for ongoing research.

In order to better understand the mechanisms of culture loss, 68 new initiations were grown in medium 16 last year (PAC Report, March 1997). Survival rates and contamination rates of these cultures were tracked for a period of 15 weeks. The data collected showed how cultures are lost over time. Cultures begin the initiation and multiplication process on the initiation medium but about 70-80% do not continue growth after the formation of a few somatic embryos. After transfer from the initiation medium some cultures require several weeks on the maintenance medium before growth again begins. Additional cultures which show growth on the maintenance medium (16) are then lost slowly from further discontinuation of growth or contamination.

In tracking last years new initiations we noted that some cultures survived in liquid medium 16 but did not survive in Gelrite-gelled medium 16. The observation suggested the hypothesis that new initiations would show increased survival if transferred to liquid medium earlier. To test this hypothesis two high school interns (Georgia Gifted and Talented Program) were given projects to each start and maintain a set of new 1997 initiations in liquid culture. The students attempted to establish 23 and 15 liquid cultures from new initiations and each succeeded in establishing 8 liquid cultures (16 total). This gives us a total liquid culture establishment and culture survival rate of 42% for 38 cultures tested.

Table 1. Loblolly pine cone collection mother trees with 1995, 1996, and 1997 initiation rates on medium 505.

Tree Identification	Initiation	Initiation	Initiation - 1997		
	1995	1996	Medium 505 %		Trial
	Media 505	Media 505 %	Individual Trials		Averages
Boisie Cascade					
BC-1		6.7%			
BC-2	10%	0.0%			
BC-3		3.2%			
BC-5		9.3%			
BC-8					
BC-9	17%	10.7%			
Champion					
C7-2			0	0	0
C7-88			3.3	0	1.7
C7-100			0		0
C8-76			3.3	6.6	4.8
C10-14			0		0
C10-38			6.7		6.7
Union Camp					
UC5-1036	32%	7.9%	3.3		3.3
UC5-1507			3.3	10	13.3
UC7-1037	10%				
UC7-1051		4.5%	3.3	3.3	3.3
UC10-1027	33%	13.8%			
UC10-5	3.3%				
UC10-33	12%				
UC11-1055		3.3%	0	6.7	0
UC11-1057		15.3%	10.3	30	30.7
UC11-1066		10.0%			
UC11-1069		4.4%			
UC11-1123			0	0	3.3
UC18-1212			3.7	26.7	40.7
Westvaco					
F-2	11%				
G-2					
H-2, 3		6.7%	26.7		26.7
I-2, 3	15%	3.3%	23.3	3.3	10
J-2, 3		7.0%	10	17.5	3.3
K-2, 3		19.0%	3.3	16.7	6.7
NC State					
NC7-1037self		0.0%	0		0
Overall	16%	7.4%			8.5%

Table 2. Media composition for initiation medium 505 and maintenance medium 16.

Components	505	16
NH ₄ NO ₃	200.0	603.8
KNO ₃	909.9	909.9
KH ₂ PO ₄	136.1	136.1
Ca (NO ₃) ₂ ·4H ₂ O	236.2	236.2
MgSO ₄ ·7H ₂ O	246.5	246.5
Mg (NO ₃) ₂ ·6H ₂ O	256.5	256.5
MgCl ₂ ·6H ₂ O	101.7	101.7
KI	4.15	4.15
H ₃ BO ₃	15.5	15.5
MnSO ₄ ·H ₂ O	10.5	10.5
ZnSO ₄ ·7H ₂ O	14.69	14.4
Na ₂ MoO ₄ ·2H ₂ O	0.125	0.125
CuSO ₄ ·5H ₂ O	0.1725	0.125
CoCl ₂ ·6H ₂ O	0.125	0.125
FeSO ₄ ·7H ₂ O	13.9	6.95
Na ₂ EDTA	18.65	9.33
Maltose	15,000	0
Sucrose	0	30,000
myo-Inositol	20,000	1,000
Casamino acids	500	500
L-Glutamine	450	450
Thiamine·HCl	1.0	1.0
Pyridoxine·HCl	0.5	0.5
Nicotinic acid	0.5	0.5
Glycine	2.0	2.0
2,4-D	0	1.1
NAA	2.0	0
BAP	0.45	0.45
Kinetin	0.43	0.43
Activated Charcoal	50	0
Gelrite	2,000	0
pH	5.7	5.7

**MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - EMBRYO
DEVELOPMENT IMPROVEMENTS BASED ON ELEMENTAL ANALYSIS OF
FEMALE GAMETOPHYTE, ZYGOTIC AND SOMATIC EMBRYO TISSUES**

**Gerald Pullman
Xiaorong Feng
Paul Montello
Yolanda Powell**

Summary

Metal analyses of full term zygotic embryo and female gametophyte tissue were compared to analyses of our most developed somatic embryos. Major differences in some elemental compositions were observed. Metal analyses were also done weekly throughout the developmental sequence for female gametophytes and zygotic embryos. These analyses showed changing compositions over time for the various elements measured. Based on these observations a series of experiments on media adjustment for each step in the somatic embryo protocol are ongoing. To date improvements in embryo yield have been documented due to reductions in boron and calcium in the development and maturation medium. Reductions in boron speeded embryo development and caused more embryos to develop. Calcium reductions also caused more embryos to be produced on development and maturation medium. A combination of ½ strength boron and ¼ strength calcium caused a repeatable statistically significant increase in yield with a marginal increase in visual embryo quality.

Over the past six months several experiments have also focused on elemental changes in the multiplication and maintenance medium. Results to date are promising and indicate that early embryo quality and subsequent cotyledonary embryo yield is improved when liquid suspension medium is modified to decrease calcium content.

Introduction

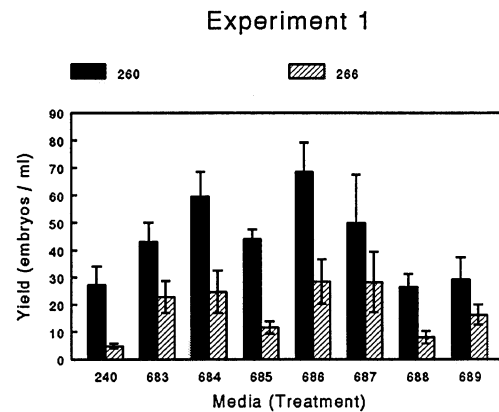
Table 1 shows a comparison of elemental analyses for zygotic and somatic tissues with the ratio of somatic / zygotics. Analyses of elemental composition of somatic embryos and comparison to zygotic targets show that somatic embryos contain 191 times the sodium, 17 times the boron, 3 times the calcium, and 1.8 times the potassium vs. the target levels of zygotic embryos. Somatic embryos are also deficient in elemental content for copper (10%), iron (21%), phosphorous (46%), magnesium (50%), and manganese (66%). Zinc and sulfur appear to be on target. Based on these findings a series of ongoing experiments was begun to test modifications in the media to produce somatic embryos which better match zygotic embryo elemental compositions. The first experiments targeted the development and maturation step but experiments are also in progress or planned to modify the initiation, maintenance and germination media.

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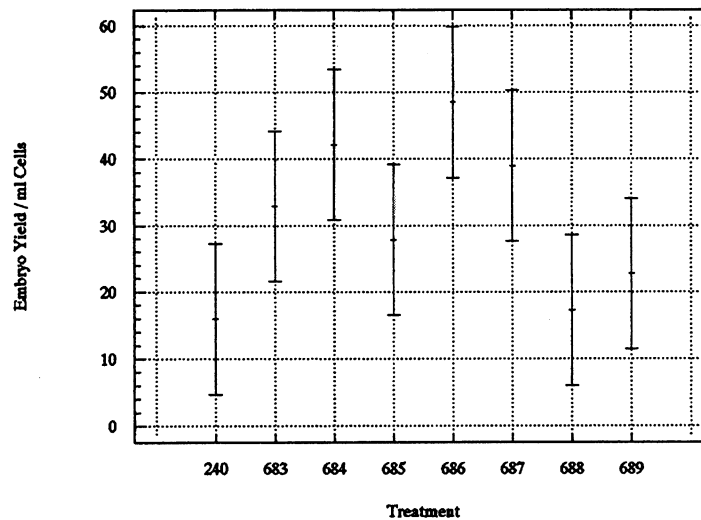
Maturation Medium Experiments

Experiment 1 (918). Our standard maintenance medium 16 was used to produce early-stage embryo suspension cultures. One ml of settled cells was plated onto sterile black filter (not containing activated carbon) papers placed onto medium 240. Modifications of 240 were tested which contained altered levels of copper, iron, or boron. Results showed statistically significant increases in embryo yield with increasing copper and with lowering boron content. Lowering boron content of the development media also speeded embryo development. Embryos produced with 1/2 the boron content appeared normal while embryos produced with increased copper were often smaller in size.

Media	Copper	Iron	Boron
240	1x	1x	1x
683	2x	1x	1x
684	5x	1x	1x
685	1x	2x	1x
686	1x	1x	1/2x
687	1x	1x	1/5x
688	1x	1x	1/10x
689	2x	2x	1/5x



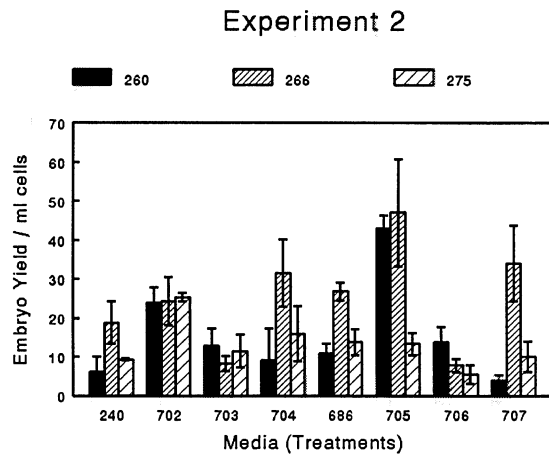
Experiment 1 - 95% Confidence
Intervals for Factor Means



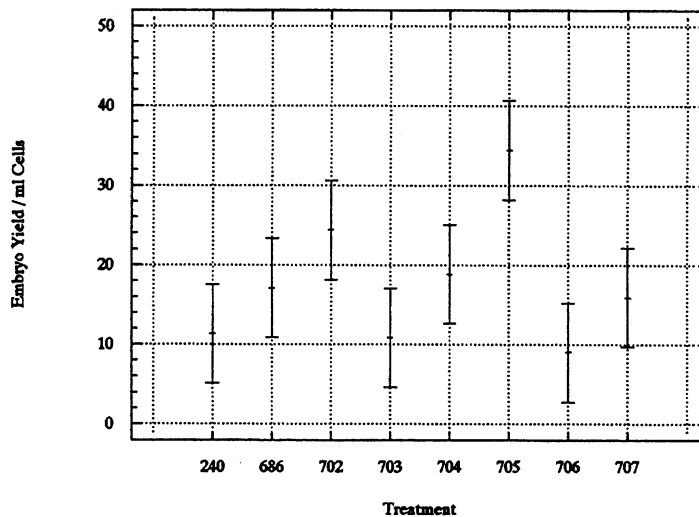
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Experiment 2 (921). With the results from experiment 1 suggesting that 1/2 boron was beneficial to speed embryo development a new experiment was set up to build upon this observation. Normal boron level or 1/2 boron were coupled with additional changes in calcium, magnesium, or phosphorous as suggested by the metal analyses. A statistically significant increase in yield over the control was seen with lowering calcium content or especially with lowering calcium combined with lowering boron. Although not significant, dry weight analyses of embryos showed that the lower boron / calcium treatment produced marginally heavier embryos.

Media	B	Ca ⁺⁺	Mg ⁺⁺	P04 ⁻
240	1x	1x	1x	1x
702	1x	0.5x	1x	1x
703	1x	1x	1.5x	1x
704	1x	1x	1x	1.5x
686	0.5x	1x	1x	1x
705	0.5x	0.5x	1x	1x
706	0.5x	1x	1.5x	1x
707	0.5x	1x	1x	1.5x



Experiment 2 - 95% Confidence Intervals for Factor Means

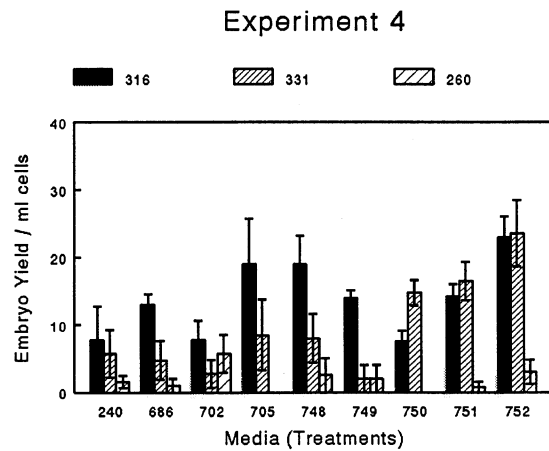


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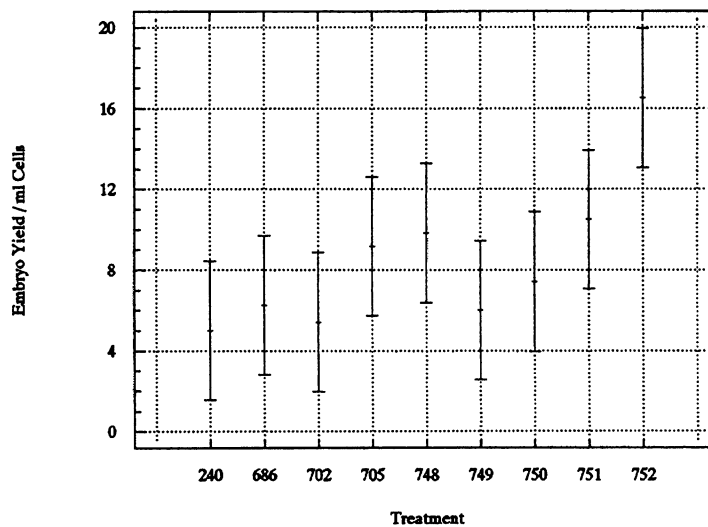
Experiment 3 (925). This experiment followed up on the observation in experiment 1 that increasing the phosphorous increased embryo yield. Three genotypes were used to test three levels of boron (1x, 0.75x, 0.5x) combined in all combinations with three levels of phosphorous (1x, 1.5x, 2x). Embryo yield was not significantly increased above the control by any combination of boron or phosphorous.

Experiment 4 (929). To confirm the statistically significant increases in yield by lowering boron and calcium together and to optimize the media composition for these elements, a experiment was designed to develop embryos using three levels of boron (1x, 0.75x, 0.5x) combined in all combinations with three levels of calcium (1x, 0.5x, 0.25x). Four genotypes were used in this experiment, one did not yield embryos.

Media	Boron	Calcium
240	1x	1x
702	1x	1/2x
748	1x	1/4x
749	3/4x	1x
750	3/4x	1/2x
751	3/4x	1/4x
686	1/2x	1x
705	1/2x	1/2x
752	1/2x	1/4x



Experiment 4 - 95% Confidence Intervals for Factor Means



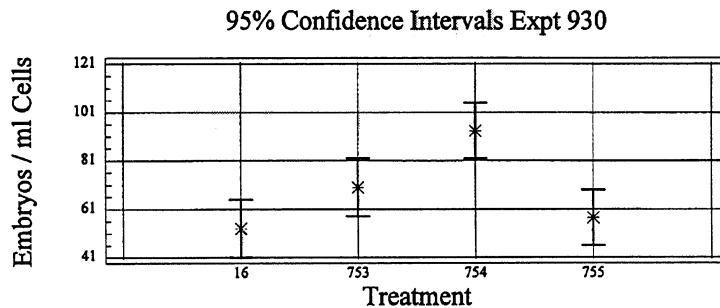
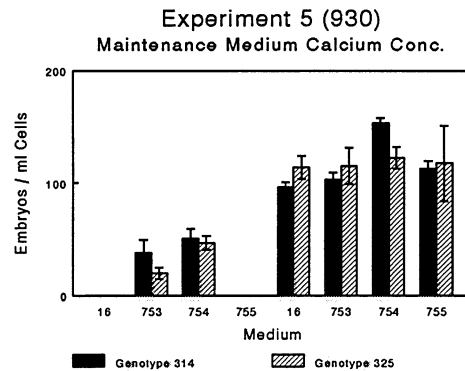
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Maintenance Liquid Suspension Culture Medium Experiments

With our analysis data of full term zygotic embryos and somatic embryos showing excess calcium and boron in somatic embryos and with the statistically significant improvements in embryo yield observed from reductions in calcium and boron in the maturation medium, we began to test the hypothesis that reductions in these elements earlier in the embryo development process would also be beneficial.

Experiment 5 (930). Our standard maintenance medium was used as a control medium or modified by the reduction of calcium content only. Three genotypes each were grown in control and modified medium and monitored weekly for ten weeks. Data on weekly settled cell volumes and early embryo stage ratings were gathered. After six and nine weekly subcultures four replications of one ml of settled cells for each genotype were plated onto sterile black filter papers placed onto medium 752 for three months. At the end of each monthly subculture filter papers and cells were transferred from the spent medium to fresh medium. Lowering calcium content to ½ strength caused a statistically significant increase in embryo yield. In addition embryo heads were observed to be larger in reduced calcium medium.

Medium	Boron	Calcium
16	1x	1x
753	1x	¾ x
754	1x	½ x
755	1x	¼ x



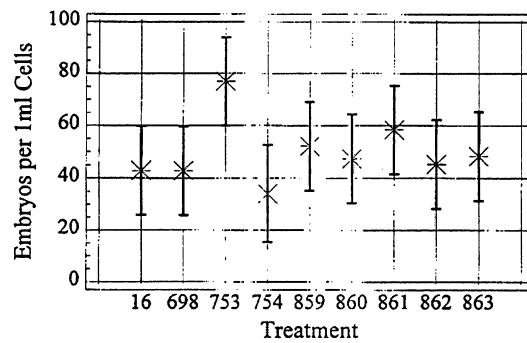
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Experiment 6 (956). An similar experiment to Experiment 4 (929) was performed for the maintenance medium. To confirm the statistically significant increase in yield from lowering calcium in the maintenance medium, an experiment was designed to develop early-stage embryos using three levels of boron (1x, 0.75x, 0.5x) combined in all combinations with three levels of calcium (1x, 0.75x, 0.5x). Three genotypes were grown in each test medium. Early-stage embryos from all genotypes and treatments were plated onto medium 752 after six and nine weeks of subculture in liquid maintenance medium.

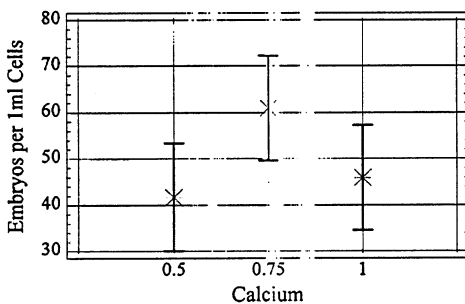
Small increases in settled cell volumes and early embryo stage ratings were observed in treatments with ¾ to ½ calcium and 1-¾ boron. Reductions in boron appeared to be less important than calcium for early-stage embryos in liquid maintenance media. The 95% confidence intervals for a combined analysis of both platings show the highest yields from medium 753. Optimal calcium levels for embryo yield appeared to be ¾ strength calcium. Varying boron level caused little change in embryo yield. Thus, maintenance medium for early-stage embryos appears to require ¾ -full strength boron and ¾ strength calcium while maturation medium requires ½ boron and ¼ calcium. These finding are consistent with metals analysis observations of decreasing calcium and boron in zygotic embryos as development progresses from early to late-stage embryos..

Media	Boron	Calcium
16	1x	1x
753	1x	¾x
754	1x	½x
859	¾x	1x
860	¾x	¾x
862	¾x	½x
698	½x	1x
861	½x	¾x
863	½x	½x

Means and 95% Confidence Intervals RP 956 Combined



Means and 95% Confidence Intervals RP 956 Combined



Means and 95% Confidence Intervals RP 956 Combined

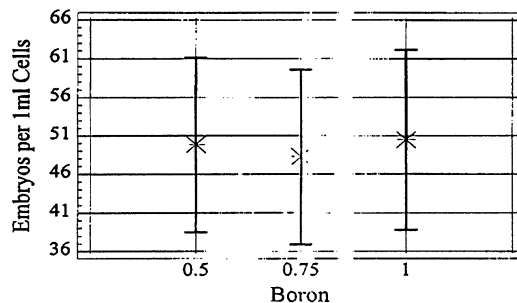


Table 1. Comparison of elemental compositions for zygotic female gametophyte, zygotic embryo, and somatic embryo tissues with along with the ratio for each element found in somatic / zygotic embryos.

Metal	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
Gametophyte	227	71	#####	3	20	149	19	12215	5388	#####	4	5132	9112	256
zygotics	81	231	#####	1.8	27.4	130	4.5	16246	2466	#####	6.9	7609	12075	182
Somatics	54	49			2.8	124	78	7449	2713		1315	3820	22204	551
Ratio Som/Zyg	0.66	0.21	#####	0	0.10	0.95	17.2	0.46	1.1	#####	191	0.50	1.8	3.0

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Table 2. Media compositions for maintenance (16), control (240), and improved (752) development and maturation media.

Components	16	240	752
NH ₄ NO ₃	603.8	200	200
KNO ₃	909.9	909.9	909.9
KH ₂ PO ₄	136.1	136.1	136.1
Ca(NO ₃) ₂ •4H ₂ O	236.2	236.2	59.05
MgSO ₄ •7H ₂ O	246.5	246.5	246.5
Mg(NO ₃) ₂ •6H ₂ O	256.5	256.5	256.5
MgCl ₂ •6H ₂ O	101.7	101.7	101.7
KI	4.15	4.15	4.15
H ₃ BO ₃	15.5	15.5	7.75
MnSO ₄ •H ₂ O	10.5	10.5	10.5
ZnSO ₄ •7H ₂ O	14.4	14.4	14.4
Na ₂ MoO ₄ •2H ₂ O	0.125	0.125	0.125
CuSO ₄ •5H ₂ O	0.125	0.125	0.125
CoCl ₂ •6H ₂ O	0.125	0.125	0.125
FeSO ₄ •7H ₂ O	6.95	6.95	6.95
Na ₂ EDTA	9.33	13.9	13.9
Maltose	0	20000	20000
Sucrose	30,000	0	0
PEG 8,000	0	130,000	130,000
myo-Inositol	1,000	20,000	20,000
Casamino acids	500	500	500
L-Glutamine	450	450	450
Thiamine•HCl	1	1	1
Pyridoxine•HCl	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5
Glycine	2	2	2
2,4-D	1.1	0	0
NAA	0	0	0
BAP	0.45	0	0
Kinetin	0.43	0	0
ABA		5.2	5.2
Activated Charcoal	0	0	0
Gelrite	0	2,500	2,500
TC Agar	0	0	0
pH	5.7	5.7	5.7

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**Mass Clonal Propagation of Improved Conifers- Embryo
Development Improvements Due To Modification of Plate Size And
Wrapping Materials**

**Jerry Pullman
Paul Montello**

**Gary Peter
Teresa Vales**

Summary

We continually strive in our program to improve embryo yield and quality. Our approach has often involved the measurement of important environmental or chemical factors during zygotic embryo development and then using this information to develop hypotheses for somatic embryo protocol improvement. Environmental factors which are very important, yet have not been thoroughly investigated in the zygotic system include gas exchange during embryo development and the nutritional reservoir available for embryo growth. Over the past six months, we began to investigate these factors with improvements culminating in two simple changes. An increase in petri plate size during the maturation step has increased embryo weight. By changing plate size from 60 X 15 mm containing 12.5 mls of medium to plates 100 X 15 mm containing 20 mls of medium, embryo fresh and dry weights increased significantly, 32% and 48% respectively. In addition, wrapping petri plates containing maturation media with a porous tape instead of the commonly used parafilm significantly increased embryo yields. This increase is shown in four separate experiments using a total of five different genotypes. Combining data from all of the experiments showed a 5.4 fold increase in embryo yield using tape as a wrapping material relative to the parafilm.

Introduction

In trying to improve our system of somatic embryogenesis in loblolly pine it was speculated that changing maturation plates from 60 X 15 mm containing 12.5 mls of media to plates 100 X 15 mm containing 20 mls of media would result in increased embryo quality and/or embryo yield. Several hypotheses were generated regarding this change. The first was that the increase in plate size would have the effect of increasing the volume of gas head space above the embryos. This larger volume increases the time it might take for inhibitory gases such as ethylene to build up to critical levels. The second hypothesis was that using the larger plates would increase the reservoir of nutrients available to the embryos as well as perhaps decrease the concentration of growth inhibitory substances or toxic by-products. These hypotheses were tested in an experiment using media 240 along with 8 different genotypes.

A long-standing procedure in our program has been the use of parafilm to wrap the petri plates. Parafilm is a relatively impermeable material resulting in small osmolality changes after one month. (Data shows a 6% increase in osmolality after one month). It is uncertain as to the amount of gas exchange that is occurring, however due to the small amount of water loss over time it was thought that the gas exchange too is small. It was hypothesized that producing an environment where atmospheric gases are allowed to diffuse in and potentially growth inhibitory gases such as ethylene are allowed to diffuse out would improve embryo maturation as evidenced

environment where atmospheric gases are allowed to diffuse in and potentially growth inhibitory gases such as ethylene are allowed to diffuse out would improve embryo maturation as evidenced

by an increase in embryo yield and/or embryo quality. The wrap materials that were chosen included a porous tape manufactured by 3M and sold by Carolina Biological Company as a product for use in plant tissue culture with a "protective barrier allowing for gas exchange" and laboratory PVC sold by Fisher which also claimed to allow gas exchange. This is a common practice in the tissue culture of dicot species. Initial experiments compared parafilm to tape. In subsequent experiments, different combinations of the three materials were used. For instance, plates were wrapped in parafilm for the first month, PVC for the second month, and tape for the third month. In addition to the gas exchange, it was hypothesized that the osmolality changes that occurred in the zygotic ovule during maturation could be mimicked by using different combinations of wrapping materials. Jerry Pullman showed in his report, IPST Technical Paper Series Number 668, that the osmolality of the ovule at early stages started high (300-600 mmol/kg) then dropped most often when the embryos were at stage 4-5 then gradually elevated to 700-800 mmol/kg. The report also showed a gradual decrease in percent water content in those ovules as the embryos moved from early stages to fully mature stage 9 embryos. Given that the PVC and tape were more porous than the parafilm, osmolality changes in the media of the maturation plates were evaluated after each months growth.

Experiment 1: Comparison of Small and Large Petri Plates

Method: Our standard maintenance medium 16 was used to produce early-stage embryo suspension cultures. One ml of settled cells was plated onto sterile black filter (not containing activated carbon) papers placed onto medium 240. Two different sizes of petri plates containing different amounts of media were used; small 60 x 15 mm plates containing 12.5 mls of media and large 100 x 15 mm plates containing 20 mls media.

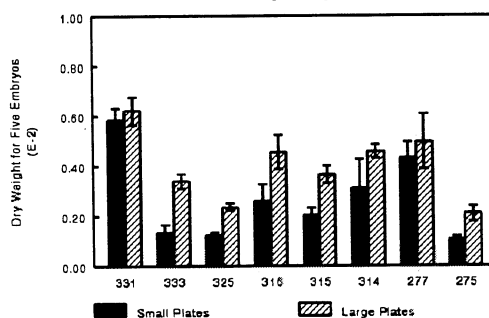
Results: Results showed statistically significant increases in fresh weight and dry weight for embryos produced on petri plates containing the larger amount of medium. Embryo yield did not change with different sized plates.

Table 1. Average Values For Embryo Yield, Fresh Weight, Dry Weight and % Water When Grown on Small vs. Large Plates.

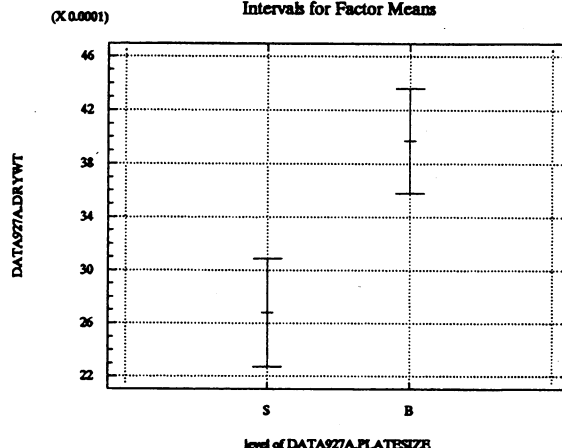
Variable	Plate Size	331	333	325	316	315	314	277	275	Average of all genotypes
Yield	Small	36.5	27.5	44.0	42.3	23.0	26.3	98.3	20.5	39.8
Yield	Large	38.3	65.0	33.5	30.0	10.2	55.0	50.0	21.0	37.9
Fresh Wt.	Small	0.0385	0.0082	0.0057	0.0150	0.0112	0.0099	0.0315	0.0049	0.0156
Fresh Wt.	Large	0.0350	0.0165	0.0081	0.0250	0.0190	0.0229	0.0297	0.0090	0.0207
Dry Wt.	Small	0.0058	0.0013	0.0012	0.0026	0.0020	0.0031	0.0043	0.0010	0.0027
Dry Wt.	Large	0.0062	0.0034	0.0024	0.0045	0.0037	0.0046	0.0050	0.0021	0.0040
Water %	Small	84.5	84.2	78.2	81.2	80.4	69.9	86.0	78.5	80.4
Water %	Large	82.2	79.6	70.0	81.5	80.6	79.8	82.8	76.7	79.2

Media	Plate Size	mls. Medium
240	60 x 15mm	12.5 mls.
240	100 x 15mm	20.0 mls.
8 Genotypes Tested		

Comparison of Small & Large Plates For Embryo Dry Weight



95 Percent Confidence Intervals for Factor Means



by an increase in embryo yield and/or embryo quality. The wrap materials that were chosen included a porous tape manufactured by 3M and sold by Carolina Biological Company as a product for use in plant tissue culture with a "protective barrier allowing for gas exchange" and laboratory PVC sold by Fisher which also claimed to allow gas exchange. This is a common practice in the tissue culture of dicot species. ()

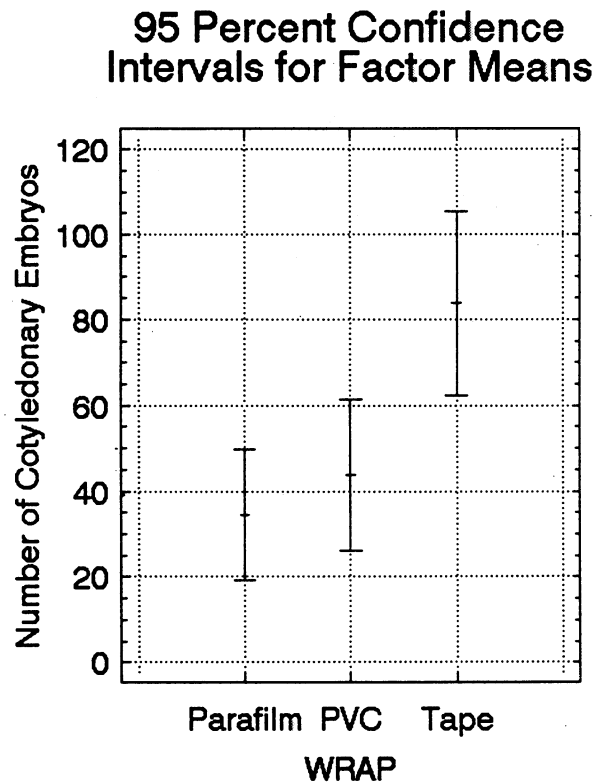
Initial experiments compared parafilm to tape. In subsequent experiments, different combinations of the three materials were used. For instance, plates were wrapped in parafilm for the first month, PVC for the second month, and tape for the third month. In addition to the gas exchange, it was hypothesized that the osmolality changes that occurred in the zygotic ovule during maturation could be mimicked by using different combinations of wrapping materials. Jerry Pullman showed in his report, IPST Technical Paper Series Number 668, that the osmolality of the ovule at early stages started high (300-600 mmol/kg) then dropped most often when the embryos were at stage 4-5 then gradually elevated to 700-800 mmol/kg. The report also showed a gradual decrease in percent water content in those ovules as the embryos moved from early stages to fully mature stage 9 embryos. Given that the PVC and tape were more porous than the parafilm, osmolality changes in the media of the maturation plates were evaluated after each months growth.



Experiment 3: Comparisons of Parafilm, Tape and PVC

Method: As in experiment 2, 1 ml of settled cells were plated on large plates containing media 752. The genotypes used in this experiment were 340 and 343. 1/3 of the plates were wrapped in parafilm, 1/3 in PVC, and 1/3 in tape. There were 5 replicates per treatment. Plates were subcultured monthly and retained the same type of wrap throughout the three month period. At three months the number of cotyledonary embryos were counted.

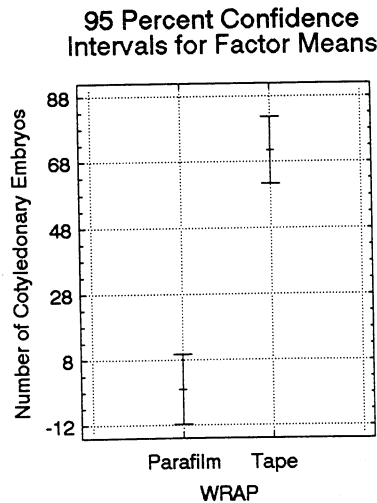
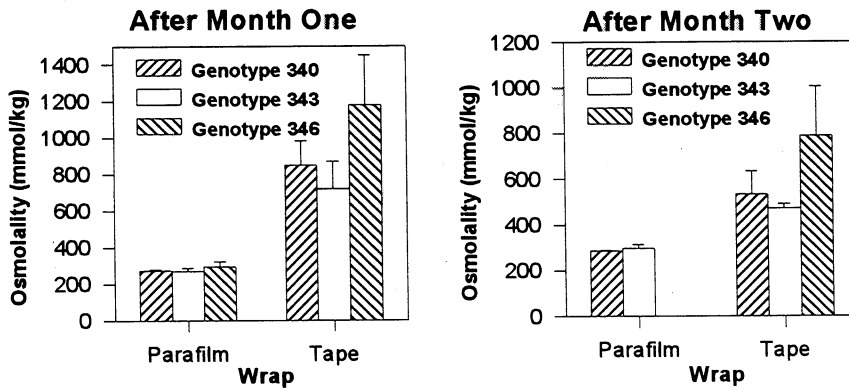
Results: Results show an average of 35 embryos in plates wrapped with parafilm, 44 with plates wrapped in PVC, and 84 in plates wrapped in tape. This represents a 1.3 fold increase over parafilm when using PVC and a 2.4 fold increase in yield over parafilm when tape is used.



Experiment 4: Comparisons of Parafilm and Tape

Method: 1 ml of settled cells from genotypes 340 , 343, and 346 were plated as in the experiments above. Plates were divided in half with one half being wrapped in parafilm and the other half being wrapped in tape. There were five replicates per treatment. The plates were subcultured monthly and retained the same type of wrap throughout the three month period. At the end of months one and two the osmolality of a representative plate from each treatment was measured using a Wescor 5500 Vapor Pressure Osmometer. After three months the number of cotyledonary embryos of each of the plates was determined.

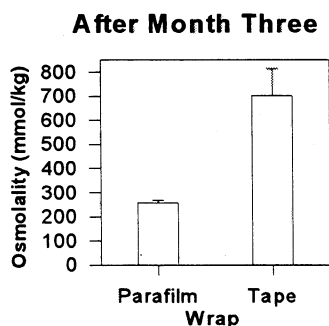
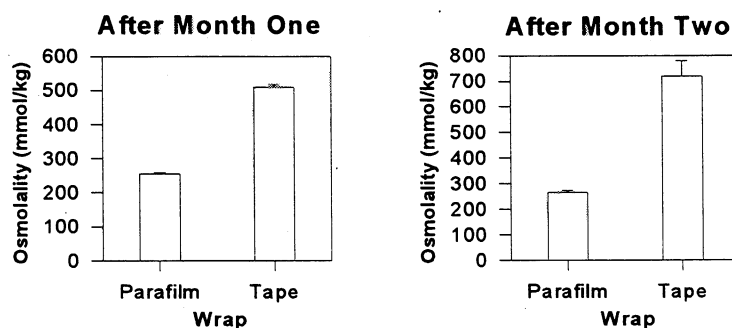
Results: In this experiment, the embryo yield for plates wrapped in parafilm was unexpectedly low. Thus the fold increase shown using the tape may be erroneously high. This experiment produced an average of .5 embryos per plate when wrapped with parafilm and 72 embryos per plate when wrapped with tape. This represents a 143 fold increase in yield with tape relative to parafilm wrapped plates. Osmolality changes after month one and two show an average osmolality of 293 mmol/kg for plates wrapped in parafilm and 598 mmol/kg for tape. More variation in osmolality exists between the plates wrapped in tape than those wrapped in parafilm. In addition, there are larger differences in osmolality between genotypes in the plates wrapped with tape compared to those wrapped in parafilm possibly due to differences in growth rates.



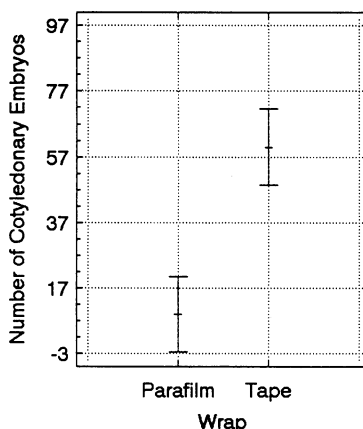
Experiment 5: Comparisons of Parafilm and Tape

Method: As in the previous experiments, 1ml of settled cells were plated on plates containing 20mls of media 752. Genotypes used in this experiment were 346 and 341. This experiment compared plates wrapped in parafilm to those wrapped in tape. The plates were subcultured monthly and retained the same type of wrap throughout the three month period. Osmolality readings were taken for one representative plate from genotype 346 per treatment after each month.

Results: Results show an average of 9 embryos when plates were wrapped with parafilm and 60 embryos on plates wrapped in tape. This is a 6.7 fold increase. The average osmolality after each month for plates wrapped in parafilm was 259 mmol/kg while those wrapped in tape were 643 mmol/kg. As in the Experiment 4, more variation in osmolality existed between plates wrapped in tape than those wrapped in parafilm



95 Percent Confidence Intervals for Factor Means

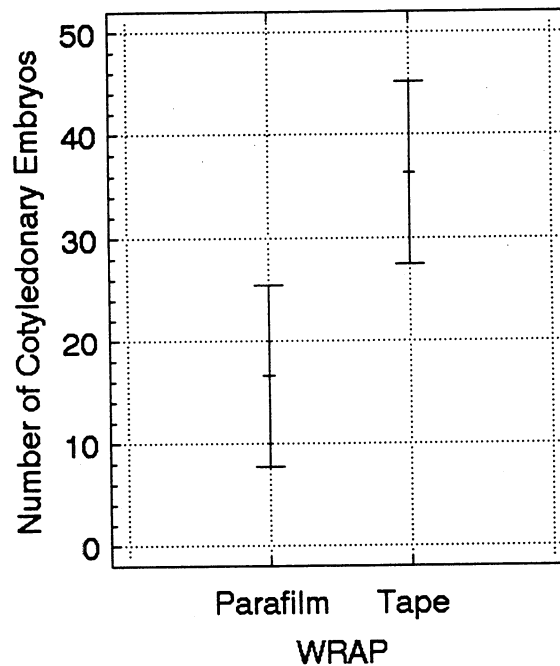


Experiment 2: Comparisons of Parafilm and Tape

Method: 1 ml of settled cells of genotypes 333 and 346 were plated onto black filter paper on top of 20 ml of media 752 in a 100 X 15mm as usual. Half of these plates were wrapped with parafilm while the other half were wrapped with tape. There were five replicates per treatment. Plates were subcultured monthly and retained the same type of wrap throughout the three month maturation period. At three months the number of cotyledonary embryos were counted. (Because genotype 346 was a more rapid developer than 333 the embryo yield data for 346 was collected after two months and compared to 333 at three months).

Results: Results show an average of 17 embryos per plate produced when parafilm was used while plates that were wrapped in tape produced 36 on average which is a 2.1 fold increase in embryo yield. This increase was shown to be statistically significant with a significance level of .004.

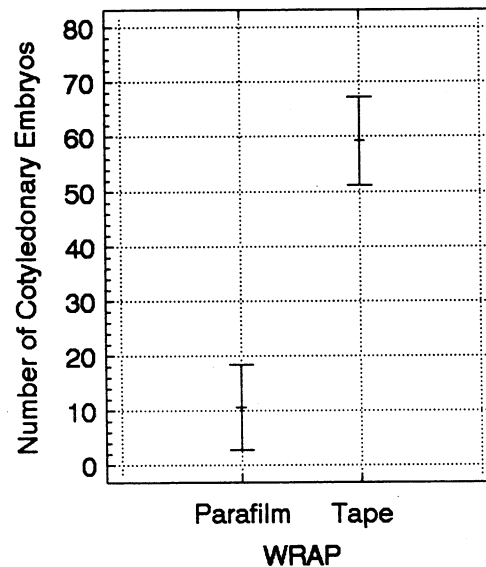
95 Percent Confidence Intervals for Factor Means



Compilation: Summary of Data From Experiments 2-5

When data from Experiments 2-5 were combined and analyzed statistically, there remained a statistically significant increase in embryo yield on plates wrapped in tape relative to those wrapped in parafilm. This reflects data from five different genotypes in four separate experiments using replicates of five. The average embryo number per plate wrapped in parafilm was 11 while the number of embryos produced on plates wrapped in tape was 56, a 5.4 fold increase. In terms of osmolality, the parafilm on average resulted in a 6% elevation after one month to about 267 mmol/kg while the PVC showed a 24% elevation to 327 mmol/kg and the tape a 62% elevation to about 653 mmol/kg. Osmolality changes using the tape and PVC were more variable between plates than the parafilm possibly due to the uneven water loss within each plate and/or the positioning of each of the plates during the one month growth period. On average, the variation in osmolality between plates wrapped in parafilm was 6% while PVC wrapped plates showed a 10% variation and tape an 18% variation. This variation needs to be reduced and will be addressed in future experiments.

95 Percent Confidence Intervals for Factor Means

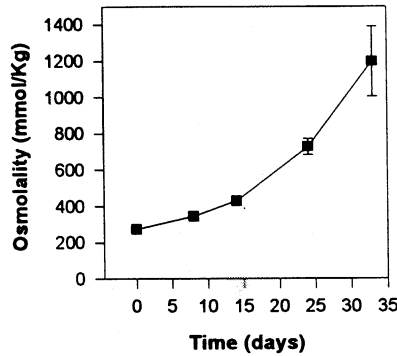


Experiment 6: Osmolality Changes Within One Month

Method: Osmolality measurements were taken from a large plate with media 752 that was wrapped with tape and which contained 1 ml of genotype 346. Readings were taken at 0 days, 8 days, 14 days, 24 days and 32 days after plating.

Results: Results show a gradual increase in osmolality starting at 273 mmol/kg and ending at 1196 mmol/kg. The rate of change appears to start slow then gain momentum after 15 days. The variation between readings on a single plate increase with time showing the greatest variation at the end of the month.





Experiment 7: Comparison of Different Combinations of Wrapping Materials

Method: This experiment included data from experiment five as well as data from additional treatments in the same experiment tried in order to mimic osmolality changes occurring in zygotic ovules. Plates were wrapped in the following combinations:

Month One	Month Two	Month Three	Abbrev
Parafilm	Parafilm	Parafilm	PPP
Tape	Tape	Tape	TTT
Parafilm	PVC	Tape	PVT
PVC	Tape	Tape	VTT
Parafilm	Tape	Tape	PTT

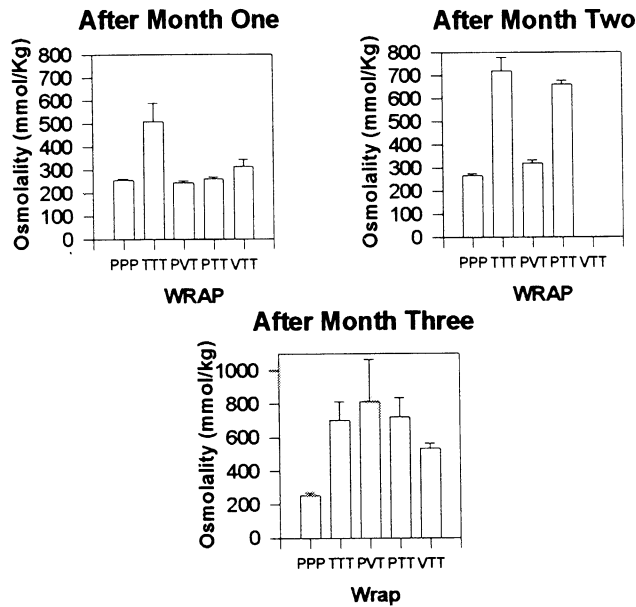
Embryo yield data was determined after three months and osmolality on plates with genotype 346 was determined after each month as well.

Results: Results show an average number of cotyledonary embryos produced after three months as follows:

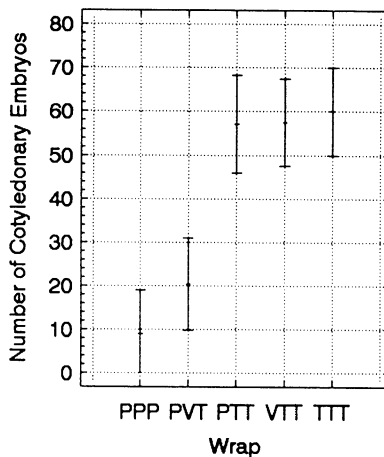
Wrap	Avg # Embryos	Fold Increase
PPP	9	
TTT	60	6.7
PVT	20.6	2.3
VTT	57.5	6.4
PTT	57.1	6.3

When statistically analyzed, it shows that the increases are statistically significant with a significance level of 000. Osmolality data shows a large variation in plates wrapped with tape especially after the third month. Osmolality changes reflect what has been seen previously for the different wraps.





95 Percent Confidence Intervals for Factor Means



Conclusions

Data has been presented that show improvements in our protocol for the maturation of loblolly pine embryos on solid media. The first improvement involved the change in petri plate size and media amount from small 60 X 15 mm plates containing 12.5 mls of media to large 100 X 15 mm plates containing 20 mls of media. Results in these experiments show improvements in the quality of the embryos, i.e. a significant increase in fresh and dry weight of the embryos. These findings present at least two hypotheses; that the increased nutrient reservoir is beneficial and that the increased gas head space is beneficial to embryo development. Based on these findings and their confirmation, our standard plating method has changed to the larger sized petri plates containing the greater amount of medium. The second potential improvement in our maturation protocol involved wrapping the petri plates with 3M Tape instead of parafilm which showed an increase the yield of embryos on the average



of 5.4 fold over parafilm. The reasoning for this improvement is at this point unclear. Hypotheses include an increase in gas exchange as well as media osmolality changes produced by the tape. Data was presented to show the effect of the tape on media osmolality. The tape unlike the parafilm causes a gradual increase over time from a starting 250 mmol/kg to osmolalities in the range of 600-800 mmol/kg in one month. Although the osmolality changes have been addressed, experiments to assess the rate of gas exchange are much harder to carry out and have not been pursued directly. Nonetheless, experiments are now in progress to determine whether ethylene plays an inhibitory role by including inhibitors of ethylene perception and biosynthesis in the maturation media. In addition, the effects of the tape wrapping during embryo maturation and the high osmolalities that result on loblolly pine somatic embryo germination are being assessed. Because of the many unknowns at this time the parafilm wrap remains in our standard protocol for the maturation of loblolly pine somatic embryos on solid media.



Table 2. Media compositions (mg/l) for maintenance (16), control (240), and improved (752) development and maturation media.

Components	16	240	752
NH ₄ NO ₃	603.8	200	200
KNO ₃	909.9	909.9	909.9
KH ₂ PO ₄	136.1	136.1	136.1
Ca(NO ₃) ₂ •4H ₂ O	236.2	236.2	59.05
MgSO ₄ •7H ₂ O	246.5	246.5	246.5
Mg(NO ₃) ₂ •6H ₂ O	256.5	256.5	256.5
MgCl ₂ •6H ₂ O	101.7	101.7	101.7
KI	4.15	4.15	4.15
H ₃ BO ₃	15.5	15.5	7.75
MnSO ₄ •H ₂ O	10.5	10.5	10.5
ZnSO ₄ •7H ₂ O	14.4	14.4	14.4
Na ₂ MoO ₄ •2H ₂ O	0.125	0.125	0.125
CuSO ₄ •5H ₂ O	0.125	0.125	0.125
CoCl ₂ •6H ₂ O	0.125	0.125	0.125
FeSO ₄ •7H ₂ O	6.95	6.95	6.95
Na ₂ EDTA	9.33	13.9	13.9
Maltose	0	20000	20000
Sucrose	30,000	0	0
PEG 8,000	0	130,000	130,000
myo-Inositol	1,000	20,000	20,000
Casamino acids	500	500	500
L-Glutamine	450	450	450
Thiamine•HCl	1	1	1
Pyridoxine•HCl	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5
Glycine	2	2	2
2,4-D	1.1	0	0
NAA	0	0	0
BAP	0.45	0	0
Kinetin	0.43	0	0
ABA		5.2	5.2
Activated Charcoal	0	0	0
Gelrite	0	2,500	2,500
TC Agar	0	0	0
pH	5.7	5.7	5.7



MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

The Effects of Increasing ABA Concentration and Extending Culture Time on Quality and Quantity of Cotyledonary Somatic Embryos of Loblolly Pine

Gerald Pullman and Xiaorong Feng

Introduction

The dry weight of late stage somatic embryos produced in our lab is similar to that of the zygotic embryos at stages 8-9.1. This dry weight is only a fraction of that of mature zygotic embryos. One of our major goals is to increase the dry weight of somatic embryos so that the quality of somatic embryos could better match to that of zygotic embryos. In prior experiment we changed the amount of two medium components, calcium and boron. When their concentrations in the maturation medium were decreased, the embryos grew faster and to a larger size than the embryos grown on control medium. The effect of different sizes of culture plates was also evaluated. There was a dry weight increase in embryos grown in large-size plates (100 X 15 mm) relative to those in regular-size plates (60 X 15 mm). However, despite these improvements, there are always some embryos in either plate that stop accumulating storage products, become green in color and begin to germinate.

ABA is a plant hormone considered to be essential for embryo maturation and germination inhibition. In several Angiosperm species, the amount of ABA increases during the middle stages of zygotic embryo development. This coincides with the acquisition of the ability of the embryos to germinate if isolated from the seeds. At late stages, although ABA usually decreases, the osmolarity in the seed will increase to keep the embryo from germinating. In loblolly pine, ABA concentration dramatically increases in the developing embryos at stages 4-7 (Data from René Howard Kapik's p.H.D. thesis). In contrast, ABA concentration in our maturation medium is a constant 5.2 mg/L for all 12 weeks of culture. This does not mimic the situation in the seeds. A previous ABA experiment conducted in our lab showed that raising ABA level in the maturation medium for the first 4 weeks decreased the embryo yield, and raising ABA level for weeks 5-8 increased embryo yield. In the current experiment, we have increased the ABA concentration and osmolarity level in the maturation medium from week 5 onwards to suppress embryo germination on this medium. This allowed us to extend the time for embryo maturation from 12 to 16 weeks, and to significantly improve the quality of cotyledonary somatic embryos of loblolly pine.

Hypotheses

1. Increasing ABA concentration in the mid- to late-stage maturation medium in combination with increased osmolarity will be able to prevent the germination activities of the somatic embryos.
2. The somatic embryos on the high ABA and osmolarity level will continue to grow and



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accumulate dry weight. This will advance the maturity of the embryos and increase the germination rate when the matured embryos are transferred to the germination medium.

Materials and methods

The standard maintenance medium 16 was used to produce early-stage embryo suspension cultures. Twenty ml of media were dispensed into 100mm x 15mm Petri dishes. Early-stage embryos from medium 16 were settled for 20 minutes after which one ml was placed on black sterile filter paper in the Petri dish. Embryos were subculture monthly. The media the embryos were transferred to during subculture were different for each treatment, and the media combinations for each treatment are listed in Table 1.

Table 1: Experiment Plan

Treatments	Week 1-4	Week 5-8	Week 9-12	Week 12-16
1 (Control)	752	752	752	752
2	752	752	796	796
3	752	796	796	796
4	752	752	797	797
5	752	797	797	797
6	752	752	798	798
7	752	798	798	798

Medium 752: ABA 5.2 mg/L, Osmolarity 250 mmo/Kg
 Medium 796: ABA 10 mg/L, Osmolarity 250 mmo/Kg
 Medium 797: ABA 5.2 mg/L, Osmolarity 400 mmo/Kg
 Medium 798: ABA 10 mg/L, Osmolarity 400 mmo/Kg

Results

1. Embryo yield:

The embryo yield is measured by embryos/plate at week 12. Because 1.0 ml suspension culture was transferred to each plate, this can also be expressed as embryos / ml suspension cells. The highest yield was produced in treatment 3 in which the embryos were cultured on medium containing 5.2 mg/L ABA for the first 4 weeks followed by medium containing 10.0 mg/L ABA for weeks 5-16 (Figure 1). At the time of switching the medium at week 5, the developmental stage the embryos were at stage 4-5. The average number of embryos of three genotypes is 83/plate in this treatment, which is 80% higher than that in the control (45/plate). The statistical analysis showed that the difference between this treatment and the control is significant (Figure 2). This result suggests that increasing ABA concentration to 10 mg/L when the embryos are at



stage 4-5 could increase embryo yield.

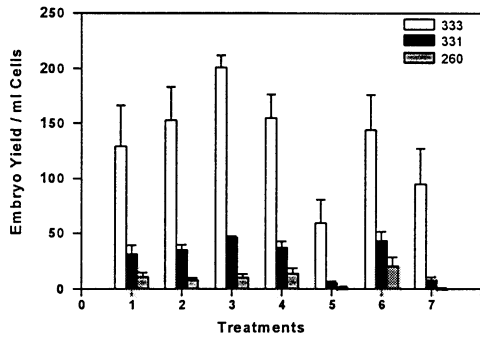


Figure 1. The yield of cotyledonary embryos measured at week 12 of culture on maturation medium.

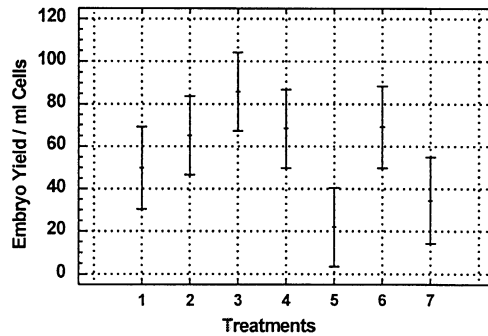


Figure 2. 95% confidence intervals for means of data in Figure 1.

2. Embryo size:

Raising ABA concentration at stage 4-5 of embryo development not only increased embryo yield, but also increased embryo size (Figure 3). When embryo length and width, as well as hypocotyl and suspensor length were measured, the results showed that both the length and width of embryos were the largest in treatment 3 (Table 2).

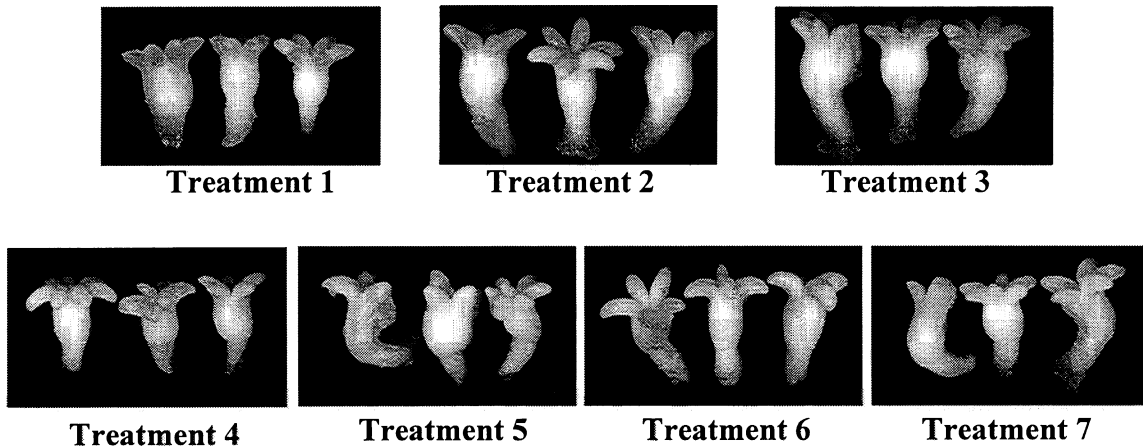


Figure 3. Pictures of embryos from different treatments listed in Table 1. The embryos were from genotype 333 cultured on maturation media for 16 weeks.



Table 2. Embryo length, width, hypocotyl length and suspensor length. Data are means in mm from genotype 333. Measurement were made after 16 weeks of culture on maturation media.

Treatments	E-length	SE	E-width	SE	hypocotyl	SE	suspensor	SE
1	1.97	0.06	1.02	0.04	1	0.04	0.61	0.03
2	2.36	0.09	1.24	0.03	1.32	0.05	0.76	0.05
3	2.54	0.08	1.29	0.05	1.28	0.07	0.95	0.04
4	2.31	0.08	1.1	0.03	1.2	0.06	0.81	0.03
5	2.18	0.07	1.2	0.05	1.21	0.07	0.76	0.05
6	2.31	0.06	1.2	0.04	1.36	0.04	0.74	0.04
7	2.3	0.09	1.23	0.05	1.29	0.05	0.79	0.05

3. Embryo dry weight:

The embryo dry weight was measured at week 12 and week 16. At week 12, the highest dry weight was obtained in

treatment 3, but the difference was not statistically significant. At week 16, high levels of ABA and osmolarity resulted in increases in embryo dry weight compared to the control. The heaviest embryos were found in treatment 5 which has a moderate ABA level (5.2 mg/L) and high osmolarity (400 mmol/Kg), although the difference was not statistically significant. The extension of culture time from 12 to 16 weeks almost doubled the embryo dry weight in all the treatments with increased ABA and/or osmolarity level (Figure 4). For example, the average dry weight of genotype 333 embryo from week 12 was 0.39 mg/embryo, and was 0.73 mg/embryo at week 16, which represents an 87% increase. This is a significant difference (Figure 5). This result suggests that extending the culture time from 12 weeks to 16 weeks could significantly increase embryo quality.

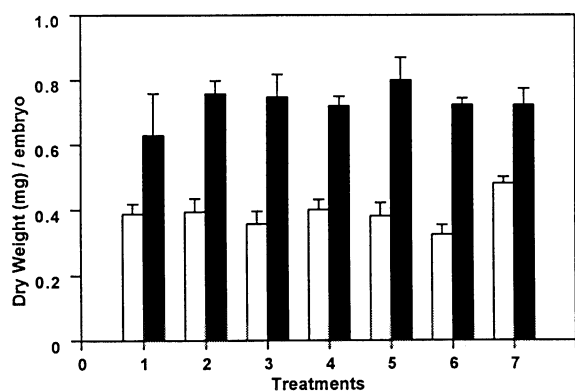


Figure 4. Dry weight of embryos cultured for 12 (blank bars) and 16 (solid bars) weeks on maturation media. Genotype 333 was used.

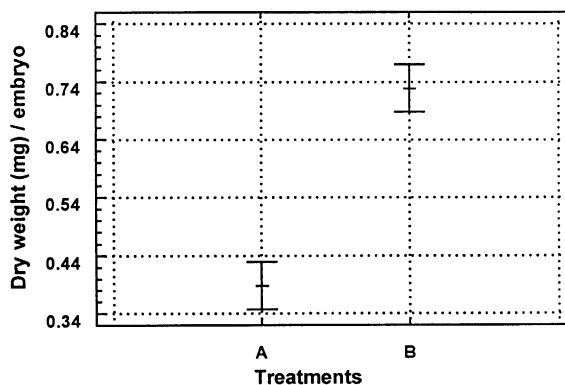


Figure 5. 95% confidence intervals for means of data in Figure 4.



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4: Germination test

One important criteria for embryo quality is the ability of the embryo to germinate when placed in suitable conditions. We tested the germination rate of the embryos from the above experiments. In the test, 10 embryos from each embryo maturation plate were transferred to germination medium, and germination was examined after 12 weeks. The results are summarized in Table 3. There were no viable seedlings produced from somatic embryos of genotype 331, however, embryos of this genotype from Treatment 3 produced more shoots. In genotype 333, on average, 4.6% of the embryos from the 12 week culture produced seedlings and 7.9% of the embryos from the week 16 culture produced seedlings. Extending the culture time greatly increased germination rate. The highest germination rate was obtained from embryos in Treatment 3. In the 12 week culture, the germination rate of embryos in this treatment was 10 times that of the control. In the 16 week culture, the germination rate in this treatment was even higher. These results suggest that increasing ABA concentration at stage 4-5 could produce embryos that are more likely to germinate.

Table 3. Germination test. The embryos from genotype 331 and 333A were cultured for 12 weeks, and genotype 333B for 16 weeks on the maturation media. The germination test was performed on germination medium for 12 weeks. Data are percentages of embryos that showed no growth, showed cotyledon elongation only, produced shoots or formed both shoots and roots .

Treatments	No growth	Cotyledon elongation	Shoot	Shoot and Root
Genotype 331				
1	32.5%	30%	37.5%	0
2	62.5%	10%	27.5%	0
3	27.5%	30%	42.5%	0
4	62.5%	27.5%	10%	0
6	73.3%	23.3%	3.3%	0
Genotype 333A				
1	15%	50%	32.5%	2.5%
2	5%	57.5%	32.5%	5%
3	0	47.5%	27.5%	25%
4	22.5%	50%	27.5%	0
5	42.5%	57.5%	0	0
6	20%	60%	20%	0
7	60%	40%	0	0
Genotype 333B				
1	0	52.5%	35%	12.5%
2	0	52.5%	42.5%	5%
3	0	44.5%	27.5%	28%
4	40%	40%	10%	10%
5	47.5%	52.5%	0	0
6	42.5%	52.5%	5%	0
7	50%	45%	5%	0



Conclusions

1. Increasing ABA concentration to 10mg/L at stage 4-5 of embryo development can increase embryo yield, size, and ability to germinate

2. Extending culture time from 12 weeks to 16 weeks on maturation medium can dramatically increase embryo dry weight and ability to germinate.

3. Combining these two treatments can improve somatic embryo quality and quantity in loblolly pine.

Table 4. Media components for maturation media.

Components	752	796	797	798
NH ₄ NO ₃	200	200	200	200
KNO ₃	909.9	909.9	909.9	909.9
MgSO ₄ •7H ₂ O	246.5	246.5	246.5	246.5
KH ₂ PO ₄	136.1	136.1	136.1	136.1
Ca(NO ₃) ₂ •4H ₂ O	59.05	59.05	59.05	59.05
Mg(NO ₃) ₂ •6H ₂ O	256.5	256.5	256.5	256.5
MgCl ₂ •6H ₂ O	101.7	101.7	101.7	101.7
KI	4.15	4.15	4.15	4.15
H ₃ BO ₃	7.75	7.75	7.75	7.75
MnSO ₄ •H ₂ O	10.5	10.5	10.5	10.5
ZnSO ₄ •7H ₂ O	14.4	14.4	14.4	14.4
Na ₂ MoO ₄ •2H ₂ O	0.125	0.125	0.125	0.125
CuSO ₄ •5H ₂ O	0.125	0.125	0.125	0.125
CoCl ₂ •6H ₂ O	0.125	0.125	0.125	0.125
FeSO ₄ •7H ₂ O	13.9	13.9	13.9	13.9
Na ₂ EDTA	18.65	18.65	18.65	18.65
Maltose	20k	20k	20k	20k
Polyethylene glycol 8,000	130k	130k	187K	187K
myo-Inositol	100	100	100	100
Casamino acids	500	500	500	500
L-Glutamine	450	450	450	450
Thiamine•HCl	1.0	1.0	1.0	1.0
Pyridoxine•HCl	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5
Glycine	2.0	2.0	2.0	2.0
BAP	--	--	--	--
IBA	--	--	--	--
Abscisic acid	5.2	10.4	5.2	10.4
Gelrite	2,500	2,500	2,500	2,500
pH	5.7	5.7	5.7	5.7



F010 MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

Optimizing Plating Methods for Somatic Embryo Yields and Automated Embryo Counting

Gary Peter
Teresa Vales

SUMMARY:

Automated counting procedures hold good potential for more objectively and more rapidly determining the number of early stage embryos that are in liquid cultures and those that mature on plates. We performed experiments to see if we could improve upon our plating procedure so that automated counting procedures can be implemented for counting cotyledonary embryos. The results show that using a vacuum method for initial plating of embryos improves their distribution such that automated counting procedures based on image analysis methods can be used successfully. In two separate experiments, the automated counting procedure when compared to our commonly used manual counting procedure differed by ~5%. When compared to the manual method, the automated method has improved objectivity and speed. We have also obtained estimates of the percentage of early stage embryos that develop into cotyledonary embryos with our current maturation system. Under our normal conditions plating 1 ml of settled volume per plate, a maximum ~8% of the embryos develop to the cotyledonary stage. However, if one half of the normal amount of embryos are plated then up to 20% form cotyledons. Thus, decreasing the number of early stage embryos plated doubles the number of cotyledonary embryos that develop.

BACKGROUND:

Initial tests to use digital cameras and image analysis software to count cotyledonary embryos were promising. The size of cotyledonary embryos and their white color against the black filter paper background made them easily observed giving a high enough contrast for image analysis software to count embryos accurately. However, when embryos were too close to one another, the embryos were not adequately resolved to obtain accurate counts. In order to implement automated counting procedures, we needed to control the number of embryos that develop, their synchrony and distribute the embryos more uniformly over the entire surface of the filter paper.

We tested whether diluting the embryos in liquid media and applying them to the filter paper with light vacuum would improve the distribution of cotyledonary embryos enough to utilize automated counting procedures. To control the number of mature embryos produced on a given plate fewer early stage embryos were plated on to each plate. The number of live embryos/ml in liquid media were counted and staged after staining with fluorescein diacetate. Counting the embryos that were to be plated gave us an estimate of the percentage of embryos that develop to the cotyledonary stage, and provides a guide for how many embryos we should place onto a plate.



MATERIALS AND METHODS:

Genotype: 346

Liquid Maintenance media: 16 (see last page for media sheet)

Maturation media: 752 (see last page for media sheet)

Maturation Conditions: Plates were wrapped with tape and subcultured monthly.

Staining for Live Embryos: Cells were stained with 0.5 ug/ml final concentration of fluorescein diacetate (FDA). Live cells convert this nonfluorescent substrate to a highly fluorescent product. The fluorescence is observed with an epifluorescence microscope equipped with a “green or FITC filter package”

Counting Procedure for Early Stage Embryos in Liquid Media: 0.5 ml of cells were placed into petri plate tops and the bottom was inverted and placed inside the top to flatten the embryos into a single plane. All of the live embryos were counted and staged by scanning the complete field with a 5X objective lens. Cells from the original settled volume were too concentrated to count; therefore they were diluted 4 fold before counting.

Plating Methods: Standard method: 1 ml of settled cells was pipetted onto black filter paper sitting on a plate containing maturation media and spread out with a pipette tip. Vacuum transfer method: Black filter paper was placed at the bottom of a vacuum filtration device and a given amount of media + cells were applied. Three different dilution's were tested to determine if better distributions and reduced plate to plate variability would be observed. A small level of vacuum was applied until the surface was dry (typically 3-8 sec), and then was released immediately. The filter paper together with the cells was placed onto a plate.

Automated Counting Procedure for Cotyledonary Embryos: The plates were placed under a black and white digital camera (Hitachi KP-M1) with 570 lines of TV resolution. A 486 computer equipped with a frame grabber board (Integral Flashpoint 128/4MB) was used to capture the images. Illumination was diffuse and from above. The brightness level needed to be adjusted to maximize the difference between the background suspensor material and the embryos. The captured images were analyzed with free software, Scion Image, which is a PC version of the free NIH Image which was developed for the Apple computers.

OBJECTIVES:

1. Develop better plating methods that will: i) allow the use of automated image analysis for counting cotyledonary embryos and ii) minimize plate to plate variation for in embryo number.
2. Test whether the removal of extra maintenance media has any effect on embryo development.
3. Learn whether we are plating the appropriate number of embryos/plate.
4. Begin to understand what percent of early stage embryos mature on plates to cotyledonary stages.



RESULTS:

Development of an Automated Plate Counting Method. To facilitate spreading over the entire surface and to test whether placing less embryos would give better distributions for automated counting we tested a vacuum application method. For the vacuum method three separate dilution's and two different amounts of embryos were plated. For the control our normal procedure was used, 1ml or 0.5 ml of cells were placed onto the filter paper and spread out over the surface.

Manual counting of the cotyledonary embryos from all the plates was done normally with the aid of a dissecting or stereomicroscope. For automated counting of cotyledonary embryos the images of the plates were captured, automatically digitized and the bright embryos counted. To obtain the best results with automated counting the quality of the image is critical. Image quality can be controlled by the light position and lamp intensity as well as with the contrast and brightness controls in the image capture program. Figure 1 shows an example of images used for automated counting. Table I shows these results from two separate experiments in which the same plates were counted both manually and with computer image analysis methods.

Figure 1

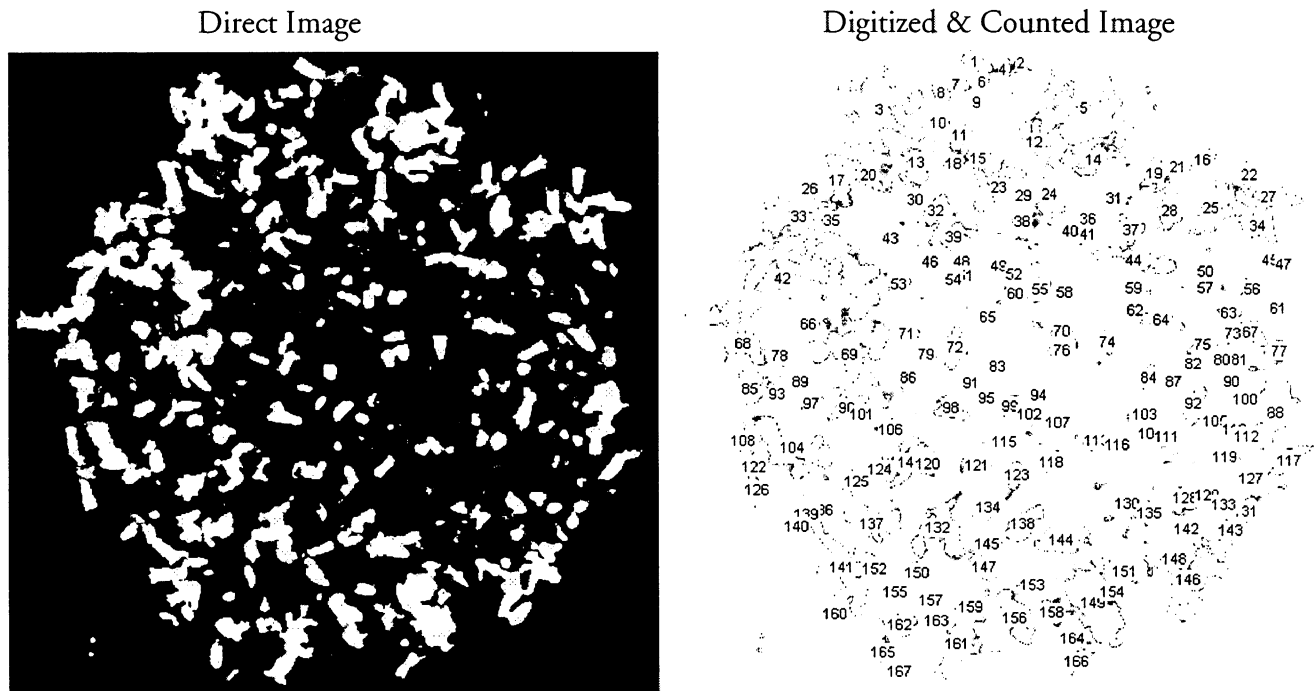


Table I. Cotyledonary Embryo Yields Determined by Manual and Automated Methods

Experiment #	Treatment: Dilution Volume (ml)	Amount (ml)	Average # Cotyledonary Embryos/Plate	Standard Deviation	Average # Cotyledonary Embryos/Plate	Standard Deviation
			Manual	Manual	Automated	Automated
1	Control	0.5	14.0	11.0	nd	-
1	Control	1.0	116	18.5	85	20.85
1	1/10	0.5	122	21.5	124	17.7
1	1/10	1.0	105	7.55	110	17.7
1	1/20	0.5	111	-	nd	-
1	1/40	0.5	99.7	17.2	95	22.7
2	Control	0.5	125	10.7	106	18.32
2	Control	1.0	89.4	14.3		
2	1/10	0.5	138	21.3	148	12.97
2	1/10	1.0	115	15.6		
2	1/20	0.5	118	6.99	126	4.55
2	1/20	1.0	82	-		
2	1/40	0.5	131	8.74	127	5.62
2	1/40	1.0	108	10.4		

Comparison between Manual and Automated Methods. Table II shows the difference between the averages obtained by manual and automated counting procedures. For experiment 1 we compared directly to the already known number obtained from manual counting with the number obtained by automated counting for each plate. The quality of the image and the parameters for counting were adjusted to obtain as close a match as possible. In contrast for experiment 2, to validate that image capture and processing parameters were well established all of the automated counts were done in a blind way with no comparison of the results till the end of the analysis.

Table II Percent Difference between Counting Methods

Experiment	Treatment	Amount (ml)	*% Difference in Avg.
1	Control	1.0	-25.9 %
1	1/10	0.5	+1.6 %
1	1/10	1.0	+4.8 %
1	1/40	0.5	-4.7 %
2	Control	0.5	-15.2 %
2	1/10	0.5	+7.2 %
2	1/20	0.5	+6.8 %
2	1/40	0.5	+3.1 %

* (manual - automated)/ manual



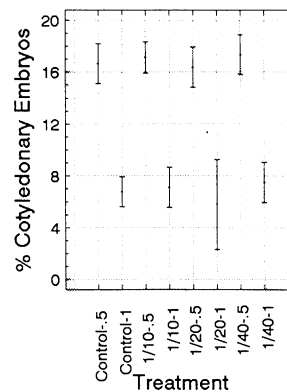
Is Vacuum Application Better for Automated Counting? Two significant problems that need to be overcome for automated counting to work well are 1) the embryos need to be well spaced and preferably not growing on top of one another, e.g. at many levels off the surface of the plate, and 2) the embryos should develop relatively synchronously into the cotyledonary stage. This second criteria does seem to be met; after two or three months of maturation most all embryos in both methods reach the cotyledonary stage.

The vacuum method is superior to our normal method; more uniform growth over the entire surface of the filter paper was consistently observed for the vacuum method. This was most evident in the control where the computer consistently underestimated the number of embryos (Table II). This low count was due to fact that the embryos were too close to be resolved. In contrast when analyzing vacuum method plates, the computer overestimated the number of embryos. Comparing the actual vacuum plates with the counted images suggests that some smaller embryos were scored by the computer that were not counted manually. To avoid counting these smaller embryos, the minimum area can be adjusted to larger size so that only bigger embryos are scored by the computer. However, in general the more significant problem even with the reduced amounts plated was that too many embryos developed such that the spacing was too close for the computer to resolve them and count them accurately.

Since embryo yields were similar between the vacuum method and our normal method, the vacuum wasn't detrimental to embryo growth nor was there a significant effect of removing the excess liquid media. In addition, the hand spreading of < 0.5 ml of embryos was less reproducible since in one of our experiments the embryos didn't develop well whereas all of the vacuum plates with 0.5ml equivalent of cells all developed well.

Parameters Limiting Automated Counting Methods. The most significant problem that still needs to be addressed is the embryo spacing. We have attempted to deal with this problem by reducing the number of embryos that are placed upon a plate. Unexpectedly, we found that as many cotyledonary embryos developed when 0.5 ml was plated as when 1 ml of cells were plated. Thus the percent of embryos that developed was effectively doubled (see next page for counting method). Figure 2 shows a statistical analysis of these results. This suggests that embryo number and or density is important to control and that some parameter, e.g., the build up of inhibitory substances or nutrient limitations somehow decrease embryo yield when too many embryos are plated. We are in the process of reducing the number of early stage embryos that are placed on each plate in a systematic way to identify the lower limit of early stage liquid embryos that need to be plated for good embryo development and for more optimal spacing for automated counting of cotyledonary embryos.

Figure 2. 95% Confidence Intervals for % of Liquid Stage Embryos that Developed to the Cotyledonary Stage on Maturation Plates

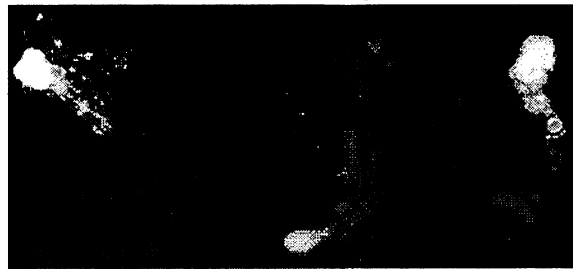


Counting Live Early Stage Embryos in Liquid Media: Many stains for viability are available. FDA has been used for the longest period. The noncharged, nonfluorescent FDA easily penetrates cells and once in the cytoplasm and organelles endogenous esterases cleave off the ester trapping the highly fluorescent acid in the cytoplasm.

Figure 3 shows a typical field of early stage embryos from liquid cultures stained with FDA.



Visible Light



FDA-Stained-Green Fluorescence

Since the early stage embryos are small, a relatively high degree of magnification is needed to confidently count embryos. This makes it impossible to observe a field that contains a significant population of embryos for counting. Thus, a larger area needs to be scanned by moving the microscope stage in a systematic way. So far, no viability stain has been identified that would permit the use of automated counting procedures. Therefore, counting and staging was done manually.

Table III shows the counting results for early stage embryos in liquid cultures for experiment 2 described above. Aliquots from the dilutions were taken before removing any cells and after. Both aliquots were stained with FDA and counted as described above.

TABLE III. Total # of Live Embryos in Liquid Cultures of Genotype 346

Dilution	stage 1	stage 1.5	stage 2	Total/ml	*Initial # Embryos/ml	*Avg. # of Embryos/ml
Before 1/4	13	68	102	370	1480	
After 1/4	15	69	103	374	1496	1488
Before 1/10	5	30	32	134	1340	
After 1/10	12	37	38	174	1740	1540
Before 1/20	2	15	10	54	1080	
After 1/20	9	21	14	88	1760	1420
Before 1/40	3	12	7	44	1660	
After 1/40	3	13	2	36	1440	1550

*The number of embryos in our original cultures



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Limitations to Counting Early Stage Embryos from Liquid Cultures. To confidently score embryos one needs to be able to clearly see both the embryo head and suspensor cells. Sometimes it is difficult to see both even after focusing through the specimen, making it hard to be completely confident in identifying all embryos. This is especially true for embryos in clumps. Thus, somewhat loose criteria were used to count so that no embryos were missed. We need to develop better markers/criteria to simplify this task. The counting itself is rather time consuming, taking at least 10 minutes per plate. Appropriate stains need to be identified so that the procedure could be more objective and ultimately be automated; ideally, stains need to be identified such that one color labels the live embryo head and another color stains the live suspensor. For example, if an antibody for a suspensor specific surface epitope was used together with a viability stain such as FDA that would label the embryo head.

CONCLUSIONS:

Objective 1: Develop better plating methods that will: i) allow the use of automated image analysis for counting cotyledonary embryos and ii) minimize plate to plate variation for replicates.

- *The vacuum method spreads the embryos out more uniformly across the surface of the filter paper and should work for automated counting once the proper number of embryos are placed onto a plate. This method did not reduce the plate to plate variation substantially.*

Objective 2: Test whether the removal of extra maintenance media has any effect on embryo development.

- *Removing the excess media 16 had no effect on embryo yield.*

Objective 3: Learn whether we are plating the appropriate number of embryos/plate.

- *We are not plating the correct number of embryos/plate for automated counting to be completely accurate due to the closeness of some embryos. However, we are close to a reasonable number.*

Objective 4: Begin to understand what percent of early stage embryos mature on plates to cotyledonary stages.

- *The percent of liquid stage embryos that develop is low overall. However, putting less embryos on a plate rather than decrease it increased the percentage that develop into cotyledonary embryos. This surprising result is being followed up by plating fewer and fewer embryos/plate to test the competency of all early stage embryos to mature and to define the practical range of early stage embryos that needs to be plated in order to achieve high percentages of embryo maturation without overloading the plates.. In addition this result highlights the need for controlling the number of early stage embryos that are plated in our experiments. In order to do this on a routine basis we need to develop better staining methods for automated counting of live embryos in liquid cultures.*



Media compositions (mg/l) for maintenance (16) and maturation media (752).

Components	16	752
NH ₄ NO ₃	603.8	200
KNO ₃	909.9	909.9
KH ₂ PO ₄	136.1	136.1
Ca(NO ₃) ₂ •4H ₂ O	236.2	59.05
MgSO ₄ •7H ₂ O	246.5	246.5
Mg(NO ₃) ₂ •6H ₂ O	256.5	256.5
MgCl ₂ •6H ₂ O	101.7	101.7
KI	4.15	4.15
H ₃ BO ₃	15.5	7.75
MnSO ₄ •H ₂ O	10.5	10.5
ZnSO ₄ •7H ₂ O	14.4	14.4
Na ₂ MoO ₄ •2H ₂ O	0.125	0.125
CuSO ₄ •5H ₂ O	0.125	0.125
CoCl ₂ •6H ₂ O	0.125	0.125
FeSO ₄ •7H ₂ O	6.95	6.95
Na ₂ EDTA	9.33	13.9
Maltose	0	20000
Sucrose	30,000	0
PEG 8,000	0	130,000
myo-Inositol	1,000	20,000
Casamino acids	500	500
L-Glutamine	450	450
Thiamine•HCl	1	1
Pyridoxine•HCl	0.5	0.5
Nicotinic acid	0.5	0.5
Glycine	2	2
2,4-D	1.1	0
NAA	0	0
BAP	0.45	0
Kinetin	0.43	0
ABA		5.2
Activated Charcoal	0	0
Gelrite	0	2,500
TC Agar	0	0
pH	5.7	5.7



**MASS CLONAL PROPAGATION OF IMPROVED CONIFERS:
FIELD ESTABLISHMENT OF SOMATIC EMBRYO
DERIVED LOBLOLLY PINE SEEDLINGS**

Jerry Pullman, Paul Montello, Xiaorong Feng, Theresa Vales,
Gary Peter, Mike Cunningham

Summary: On January 22, 1997 thirty five somatic embryo derived loblolly pine seedlings, initiated in summer 1994, from open pollinated ovules from tree UC10-1003 were established in a field plot at the Union Camp Ogeechee Forest in Tattnall County Georgia.

Loblolly pine seeds that originated from tree UC10-1003 of the Union Camp Corporation were initiated during 1994 initiation trials. The somatic embryos that resulted subsequently were allowed to undergo conversion and germination. The seedlings spent approximately 1 year in the greenhouse. During the winter of 1996/97 35 of these seedlings were delivered to the Union Camp Bellville Georgia location. On January 22, 1997 they were established in the field. The study was laid out in four rows. Rows 1-3 have 9 trees whereas Row 4 has 8 trees. The spacing between seedlings is 10'x6'. The study plot is marked with a post at each corner and a flag pin at each tree.

On October 2, 1997 the trees displayed 100% survival in the field. The average height of the 35 trees were 1.9 feet. The tallest tree was 2.6 feet and the shortest was 1.2 feet (photo taken late September 1997).

As of March 1, 1998 the pine seedlings had overwintered the relatively mild winter without any problems and were expected to flush soon. In preparation to meet our goal of placing 50 somatic embryo derived seedlings for each of five genotypes in the field we placed mature embryos on germination medium and collected germinants showing shoot and root growth. Following germination, embryos were transferred to soil in magenta boxes and watered every two weeks. Our preliminary results are as follows:

Genotype	Alive in Soil	Growth in Soil	Plants in Field
195	12	12	35
333	34	11	
346	22	14	
344	152	152	

We would like to thank Mike Cunningham, Randy Purvis, and Jerome Martin at Union Camp for the establishment of these seedlings.



MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - ELEMENTAL ANALYSIS OF ZYGOTIC FEMALE GAMETOPHYTE AND EMBRYO TISSUES

**Gerald Pullman
Mike Buchanan
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Xiaorong Feng
Greg Elay**

Summary

Full term seeds from loblolly pine were dissected to remove the seed coat, integument, and nucellus and divided into the female gametophyte and embryo tissue. Tissue was analyzed in replicate for major and minor elements by use of Inductively Coupled Plasma Emission Spectroscopy (ICPES). Five sources of seed were analyzed in order to determine an approximate range of natural elemental composition. Variation between the five seed sources was minimal providing elemental composition targets for somatic embryos. Analyses of elemental composition of somatic embryos and comparison to zygotic targets show that somatic embryos contain 250 times the sodium, 17 times the boron, 3 times the calcium, and 1.8 times the potassium vs. the target levels of zygotic embryos. Somatic embryos are also deficient in elemental content for copper (10%), iron (21%), phosphorous (46%), magnesium (50%), and manganese (66%). Zinc and sulfur appear on target. Microelements such as nickel, cobalt, and molybdenum were below detection levels in somatic and zygotic embryos. Analyses of developing zygotic embryos and female gametophyte tissues show significant changes in elemental composition over time. Phosphorous, potassium, magnesium, calcium, boron, sodium, and zinc contents in the female gametophyte are highest during early seed development. Sulfur, iron, and copper contents are relatively constant during female gametophyte growth. Zygotic embryo tissue showed a manganese peak at stages 1-2. Peaks in boron and sodium occurred at zygotic embryo stage 3-4. Embryo tissue peaks in phosphorous, potassium, calcium, and zinc, occurred at stage 5-6. Iron showed an initial peak at stage 5-6 followed by a dip and then increased until embryos stopped growth. Magnesium rose slightly throughout embryo development and sulfur and copper were fairly constant through embryo development. Changes were made in the metal content of the tissue culture medium based on the analysis data obtained. Statistically significant increases in embryo yield occurred in the modified maturation and development medium. Metal analysis of embryos grown on modified medium shows changes in metals content towards the desired target levels.

Introduction

The nutritional, osmotic, and hormonal environments surrounding an embryo are well known to control embryo growth. Optimization of these environments is critical for the growth and development of high quality, vigorous somatic embryos. In optimizing the nutritional environment for somatic embryo development, we propose that analysis of the elemental contents of the female gametophyte and the zygotic embryo will provide models and targets for the development medium and somatic embryo elemental compositions respectively.

Following is a tentative step by step plan of how we plan to use analysis information of zygotic and female gametophyte tissue to improve our somatic embryo protocol. So far, we have completed the steps 1,2 and have some data for step 4. Experiments are in progress for steps 3 and 5.

Step 1. The first phase of this research is to determine how wide or narrow the natural or acceptable range of elemental composition is for female gametophyte and embryo tissue. We decided to initially use full term loblolly pine seed due to its availability and ease in obtaining the necessary sample weights for analysis. This data provides elemental targets for the somatic embryo and potentially for the medium.

Step 2. Analyze somatic embryos for elemental composition. Use somatic embryos as close to maturation as we can currently grow. Compare to target range for each element in analysis.

Step 3. Modify development and maturation medium based on review of the elemental composition data. Grow new somatic embryos on modified medium, observe somatic embryos for morphological improvements, analyze somatic embryos for new elemental composition and fit to target range.

Step 4. Analyze female gametophyte and zygotic embryos for each developmental stage to provide a time course of elemental composition during embryo and female gametophyte developments. (Note, some methods development is necessary to analyze the small amounts available of very early stage zygotic embryos. Example, twenty stage one embryos have a dry weight of approximately 0.3 mg)

Step 5. Modify initiation, maintenance, and maturation medium based on the pattern of elemental change in developing female gametophyte and zygotic embryo tissues. Observe somatic embryos for morphological improvements, analyze somatic embryos grown in modified medium for new fit to elemental target range.

Materials and Methods

Step 1. (Presented in the 1997 Spring PAC Report.) Five sets of cones or seed lots of ½ sib materials from four locations and seed orchards were analyzed for embryo and female gametophyte elemental composition. In order to make seed and embryo dissection easier, cones were requested at the end of seed development just prior to the seed drying process. With seed collection just prior to drying any potential elemental loss due to imbibition was eliminated and seeds were easier to open and remove embryos. Seed collections of 50-100 mg dry weight were obtained as follows.

BC-1	Boisie Cascade 1995 seed produced in a seed orchard near Lake Charles, Louisiana.
UC5-1036	Union Camp 1995 seed produced in a seed orchard near Bellville, GA.
UC10-1003	Union Camp 1995 seed produced in a seed orchard near Rincon, GA.
UC10-14	Union Camp 1995 seed produced in a seed orchard near Rincon, GA.
7-56	Westvaco 1995 seed produced in a seed orchard near Summerville, SC

Collected embryos were dried overnight at 70 °C and stored in a freezer. Pre-dried specimens of Loblolly Pine embryos and gametophytes were submitted for elemental analysis in individually labeled polyethylene micro-centrifuge tubes. To enable these samples to be analyzed for trace metals by Inductively Coupled Plasma Emission Spectroscopy (ICPES), the constituent metals present in the solid sample material were dissolved in an aqueous acid solution. The preparation procedure followed is outlined below:

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1. An aliquot (approximately 50 mg) of pre-dried sample, was weighed into a new, labeled, graduated, screw-cap, polyethylene centrifuge tube. The weight of the sample was recorded to the nearest 0.1 mg.
2. Five milliliters (5.0 ml) of high purity concentrated Nitric Acid (EM Science TracePur Plus Instrumental Grade) were added to each tube, then capped, and allowed to stand at room temperature for six hours in a fume hood.
3. The tube was uncapped and two milliliters (2.0 ml) of high purity 30% Hydrogen Peroxide (J.T. Baker Ultrex Ultrapure Reagent Grade) were added. The tube was capped, inverted twice to mix the contents, and vented to release any evolved oxygen. The cap was loosely screwed on the tube to prevent pressure buildup from evolved oxygen. Each sample was allowed to digest at room temperature for 24 hours in a fume hood.
4. At the end of the digestion period, ultrapure reagent grade deionized water (ASTM Type I water), was added to each tube to bring the total solution volume to 10.0 ml.
5. Prior to analysis, each sample was filtered through a 0.45 μm membrane syringe filter.

Analysis of the sample digests was conducted on a Perkin Elmer Optima 3000 DV ICP Emission Spectrometer. This instrument, equipped with an autosampler and integral computer workstation, is configured to detect up to 30 elements simultaneously in less than five milliliters (5 ml) of sample solution. The principle of analysis is the detection of characteristic ultraviolet and visible light emissions from metallic elements subjected to a high temperature argon plasma torch. To improve instrument performance, a Yttrium internal standard is added to each sample, standard and blank to compensate for small variations in sample flow rate, sample viscosity and acid concentration as well as to assist in the identification of potential interferences. Quantification of the metallic analytes in the sample is based on measuring specific wavelength intensities for each element and comparing these results to multi-point calibration standards analyzed in the same manner.

The instrument is calibrated daily with three multi-component standards and a blank. A series of verification standards, interference check solutions and blanks are analyzed and evaluated before any samples are analyzed. At a frequency of every ten samples, a calibration verification standard and blank are analyzed. Acceptance criteria for each standard, blank and sample measurement are defined and used to accept or reject results.

Step 2. (Presented in the 1997 Fall PAC Report.) Somatic embryos from genotype 195 (an initiation from immature seed from tree UC 10-1003) were produced in maintenance medium 16 and embryos grown on medium 240. Somatic embryos were collected at the end of the development period on medium 240; corresponding in appearance to zygotic stages 8-9.1. Somatic embryos were collected from three different batches of embryos over a period of three months, each batch was produced from the same genotype on the same maintenance and development medium. Each of these three replicates of somatic embryos contained 30-50 mg dry weight of embryo. Metals analysis were done as described for step 1.

Step 3. Data was presented in the 1997 Fall PAC Report showing statistically significant embryo yield improvements resulting from analysis-based modifications to the maturation and development medium. A series of experiments were presented which produced an improved maturation and development medium (medium 752) containing $\frac{1}{2}$ of the boron and $\frac{1}{4}$ of the calcium levels.

Over the past six months embryos were grown on metals modified medium and removed for analysis. Our original hypothesis was that the improved development medium, containing altered metal components, would produce embryos which better resembled the target zygotic embryo metal levels. We decided to try a small test and analyze a small amount of tissue without replicates for a preliminary view. Small amounts embryos were collected from control and modified media, dried overnight at 70 °C. Approximately 5-10 mg of dry wt. tissue were prepared as described in step 4 below. Operation of the Perkin Elmer Optima 3000 DV ICP Emission Spectrometer was described in steps 1 and 4. Again, due to the low amounts of tissue available for analysis the standard operating mode was modified. To yield the lowest possible detection limits the instrument was operated in the "axial" view mode which effectively increases the path length of the spectroscopic measurement.

Step 4. During 1994 loblolly pine cones were collected weekly throughout the sequence of embryo development from Union Camp tree UC5-1036, located in a seed orchard near Bellville, GA. Cones were shipped on ice to IPST and received within 24-48 hours of collection. Cones were opened and seeds collected for isolation of embryos. Seeds were cracked using a hemostat, pried open with the aid of a scalpel, and the integument and nucellus tissue removed from the ovule. The female gametophyte was slit, pried open and the dominant embryo or mass or embryos removed. Individual embryos were quickly observed through a dissecting microscope, evaluated for stage of development (Pullman & Webb, 1994), sorted by stage, and placed in vials partially immersed in liquid nitrogen. Stage 9 embryos were also categorized by the week they were collected; 9.1 (Stage 9, week 1) 9.2 (Stage 9, week 2) etc. Staged zygotic embryos were then placed in a glass vial partially immersed in liquid nitrogen. Frozen embryos were stored at -70 °C. Samples were dried overnight at 70 °C. Due to the small amounts of embryo tissue available from stages 1-8, the analysis method was modified as follows.

1. An aliquot of pre-dried sample, was weighed into a new, labeled, graduated, screw-cap, polyethylene centrifuge tube. The weight of the sample was recorded to the nearest 0.1 mg. Weights ranged from a low of 0.5 mg for a mass of early stage embryos to 79.4 mg for later stage embryos or female gametophyte.
2. One half milliliter (0.5 ml) of high purity concentrated Nitric Acid (EM Science TracePur Plus Instrumental Grade) was added to each tube, then capped, and allowed to stand at room temperature for 18 hours in a fume hood.
3. The tube was uncapped and one milliliter (1.0 ml) each of high purity reagent water and 30% Hydrogen Peroxide (J.T. Baker Ultrex Ultrapure Reagent Grade) were added. The cap was loosely screwed on the tube to prevent pressure buildup from evolved oxygen. Each sample was allow to digest at room temperature for 24 hours in a fume hood.
4. The tube was uncapped and one milliliter (1.0) of high purity concentrated hydrochloric acid (J. T. Baker Instra-Analyzed Reagent Grade) was added. Each sample was allowed to digest an additional 24 hours. At the end of the digestion period, ultrapure reagent grade deionized water (ASTM Type I water), was added to each tube to bring the total solution volume to 10.0 ml.

5. Prior to analysis, each sample was filtered through a 0.45 µm membrane syringe filter.

Operation of the Perkin Elmer Optima 3000 DV ICP Emission Spectrometer was described in step 1 above. Due to the low amounts of tissue available for analysis the standard operating mode was modified. To yield the lowest possible detection limits the instrument was operated in the “axial” view mode which effectively increases the path length of the spectroscopic measurement.

Results and Discussion

Step 1. The elemental analyses for zygotic embryo are very similar for the five seed sources tested. Summaries of the averages per seed source and for all replications are shown for zygotic embryos in Table 1. Analyses for female gametophyte tissue is also similar for the five seed sources and is shown in Table 2. Standard errors for zygotic embryo elemental variation between all of the replicates for Mn, Fe, Cu, Zn, P, S, Mg, and K are less than 4% of the mean values (Table 1). Nickel, B, Na, and Ca show greater variation with standard errors ranging from 5-16% of the mean (Table 1). Standard errors for female gametophyte elemental variation between all replicates for Fe, Ni, Cu, Zn, P, S, Mg, and K are less than 4% of the mean values (Table 2). Manganese, B, Na, and Ca show greater variation with standard errors ranging from 5-8%.

Individual replicate analyses for zygotic embryos per site are shown in Tables 3-7. Individual replicate analyses for female gametophyte tissue per site are shown in Tables 8-12. Elemental concentrations detected for Cobalt, Nickel, Molybdenum, and Sodium were sometimes below the accurate detection limits of the equipment and values for these replicates are shown as <.

Elemental concentrations of zygotic embryo and female gametophyte tissues were often different. Ratios of elemental compositions, on a dry weight basis, are shown in Table 13.

Embryos contained low contents of Mn, B, and S, suggesting that these elements are selectively excluded from the embryo. Similar contents of Ca, Ni, Zn, and Cu were found in embryo and female gametophyte tissue suggesting that these are taken into the embryo by diffusion. Greater concentrations of P, K, Mg, Na, and Fe were found in the embryo compared to female gametophyte tissue suggesting that these elements are actively taken up by the embryo.

Overall, the similarity in analyses of zygotic embryo tissues suggest that the mean elemental compositions of zygotic embryos provide reasonable targets for the elemental composition of somatic embryos.

Step 2. The analyses for somatic embryos are shown in Table 14. Table 15 shows a comparison of average zygotic female gametophyte, average zygotic embryo, Clone 195 somatic embryo and a ratio of somatic/zygotic. These comparisons clearly show that our somatic embryo is way off for certain elements and on target for others.

Somatic embryos show major differences in some elements compared to zygotic embryos. The greatest difference is for Boron. Somatic embryos contain 250 times as much sodium as zygotic embryos, 17 times as much boron, three times the target amount of calcium, and 1.8 times potassium. These

observations suggest the hypothesis that decreasing sodium, boron, calcium, and potassium in the maintenance or development media will improve embryo growth and development. On the other side, some elements were low in the somatic embryos. Somatic embryos contained only 10% of the copper contained in zygotic embryos, 21% of the iron, 46% of the phosphorous, 50% of the magnesium, and 66% of the manganese. These observations suggest that these elements should be increased in the maintenance or development media. Other elements such as zinc, and sulfur appear to be on target. Microelements such as nickel, cobalt, and molybdenum were present in zygotic and somatic embryos at amounts below detection levels of the equipment used.

Step 3. The elemental compositions of somatic embryos grown on control and modified medium are shown in Table 16. This preliminary information reveals that somatic embryo metal content is moving towards the target levels. These results are promising, embryos grown on reduced boron or calcium contain target mineral contents that are more similar to the target levels contained in zygotic embryos. Embryos grown on decreased calcium and boron contain less calcium and boron. Embryos grown on increased iron contain higher levels of iron. Our next step is to complete a larger replicated experiment with several genotypes. Additional experiments are ongoing for alteration of the maintenance and development media based on the analyses in steps 1 and 2.

Step 4. Elemental analyses throughout the sequence of development for female gametophyte and embryo tissues are shown in Figures 1-8. The female gametophyte tissue feeds the embryo during development and during germination. Elemental contents of the female gametophyte change over time (Figures 1-4). Phosphorous, potassium, magnesium, calcium, boron, sodium, and zinc contents in the female gametophyte are highest during early seed development. Sulfur, iron, and copper contents are relatively constant during female gametophyte growth. Female gametophyte elemental concentrations were more closely related to the date of tissue collection than to the stage of embryo contained within.

Zygotic embryo elemental contents also change over time (Figures 5-8). Manganese showed a peak at stages 1-2. Peaks in boron and sodium occurred at stage 3-4. Since embryos were collected in sodium borosilicate glass vials during 1994, it is possible that some container contamination may be present. However, all samples were treated similarly yet distinct peaks in boron and sodium are clearly present. Embryo collections for later years have used plastic cryostorage vials. Peaks in phosphorous, potassium, calcium, and zinc, occurred at stage 5-6. Iron showed an initial peak at stage 5-6 followed by a dip and then increased until embryos stopped growth. Magnesium rose slightly throughout embryo development and sulfur and copper were fairly constant through embryo development. In evaluating these mineral changes it is important to consider that early embryo stages 1-4 contain decreasing masses of suspensor tissue. Analyses of stage 1 embryo would consist mostly of suspensor tissue.

Table 1. Summary of averages of replicated elemental analysis of zygotic embryos collected from loblolly pine seeds grown on different mother trees and in different locations.

Tree	Location	Rep	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
BC-1	Lake Charles, LA	5	89.4	258	3.4	28.7	146	6.0	17053	2747	7671	12678	173			
UC5-1036	Bellville, GA	4	90.6	279	1.0	24.6	133	8.7	17432	2619	8122	13092	275			
7-56	Summerville, SC	3	66.4	221	1.0	22.7	113	1.2	15440	2242	7.1	7352	140			
UC10-14	Rincon, GA	2	76.7	181		31.0	113	2.2	14883	2312	6.6	7283	174			
UC10-1003	Rincon, GA	1	82.1	215	<.40	<.59	30.0	147	<.36	16421	2410	<.52	146			
Mean		15	83.0	243	####	2.1	26.8	132	6.2	16500	2531	####	192			
Std Error		15	3.0	9.7	####	0.3	0.8	4.3	1.0	282	57	####	28			
Std			0.04	0.04	####	0.16	0.03	0.03	0.16	0.02	0.02	####	0.15			
Er/Mean																

Table 2. Summary of averages of replicated elemental analysis of female gametophyte tissue embryos collected from loblolly pine seeds grown on different mother trees and in different locations.

Tree	Location	Reps	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
BC-1	Lake Charles, LA	5	243	74	3	20	127	28	12415	5867	5019	9410	203			
UC5-1036	Bellville, GA	4	233	82		22	143	21	13525	5547	5453	9744	344			
7-56	Summerville, SC	3	121	53		16	126	17	10804	4633	4	4621	270			
UC10-14	Rincon, GA	2	251	79		18	155	19	11981	5430	4	5435	150			
UC10-1003	Rincon, GA	1	287	66	<.35	<.55	21	193	12	12348	5465	<.45	311			
Mean		15	221	72	####	2.6	20	139	22	12327	5450	####	254			
Std Error		15	14.8	3.1	####	0.1	0.6	4.9	1.5	256	120	####	21			
Std Er/Mean			0.07	0.04	####	0.03	0.03	0.04	0.07	0.02	0.02	####	0.08			

Table 3. Elemental analysis (mg/Kg) for zygotic embryos of BC-1 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
BC-1	89.3	283.4	<0.43	3.5	29.2	154.7	7.7	17302	2715	<0.55	<3.44	7740	12675	171.9
BC-1	87.9	249.8	<0.43	4.1	28.9	139.5	6.9	16806	2711	<0.55	<3.45	7617	12708	200.7
BC-1	89.1	271.3	<0.40	2.3	30.4	160.6	4.2	17543	2824	<0.52	<3.24	7924	13009	137.0
BC-1	97.8	266.3	<0.40	3.3	28.4	147.0	3.1	17525	2793	<0.51	<3.22	7823	13050	180.2
BC-1	83.2	218.3	<0.42	3.9	26.7	129.5	8.1	16091	2692	<0.54	<3.40	7253	11948	175.6
Mean	89.4	257.8		3.4	28.7	146.3	6.0	17053	2747			7671	12678	173.1
Std Error	2.4	11.3		0.3	0.6	5.5	1.0	274.9	25.8			116.1	197.8	10.3

Table 4. Elemental analysis (mg/Kg) for zygotic embryos of Union Camp 5-1036 from full term cones collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC5-1036	83.2	267.3	<0.42	1.4	24.6	129.4	8.0	16961	2539	<0.54	<3.35	7958	12791	150.6
UC5-1036	97.6	291.6	<0.42	0.9	24.6	126.6	3.1	17630	2652	<0.54	<3.39	8158	13196	188.9
UC5-1036	100.6	292.7	<0.38	0.7	25.2	143.7	14.3	17700	2595	<0.48	5.1	8273	13120	574.1
UC5-1036	81.2	265.5	<0.42	1.1	24.0	130.5	9.3	17435	2688	<0.54	<3.37	8098	13259	185.1
Mean	90.6	279.3		1.0	24.6	132.5	8.7	17432	2619			8122	13092	274.7
Std Er	4.9	7.4		0.2	0.2	3.8	2.3	167	33			66	104	100.2

Table 5. Elemental analysis (mg/Kg) for zygotic embryos of Westvaco 7-56 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
7-56	73.0	227.6	<0.40	1.4	24.8	118.3	1.2	16132	2340	<0.51	8.4	7707	12232	100.7
7-56	60.6	210.5	<0.38	0.7	21.5	108.9	<0.35	14617	2122	<0.49	7.2	6948	10732	115.4
7-56	65.8	223.8	<0.41	<0.61	21.9	112.5	<0.38	15572	2265	<0.53	5.8	7401	11691	203.9
Mean	66.4	220.7		1.0	22.7	113.2	1.2	15440	2242		7.1	7352	11552	140.0
Std Er	3.6	5.2		0.3	1.1	2.7	0.0	442	64		0.7	220	438	32.2

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Table 6. Elemental analysis (mg/Kg) for zygotic embryos of Union Camp 10-14 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC10-14	73.5	179.6	<0.40	<0.58	31.2	115.3	<0.36	15063	2312	<0.51	<3.18	7338	11328	157.1
UC10-14	79.9	181.5	<0.41	<0.60	30.8	110.5	2.2	14703	2312	<0.53	6.6	7228	11109	191.8
Mean	76.7	180.6			31.0	112.9	2.2	14883	2312		6.6	7283	11219	174.5

Table 7. Elemental analysis (mg/Kg) for zygotic embryos of Union Camp 10-1003 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC10-1003	82.1	214.8	<0.40	<0.59	30.0	147.5	<0.36	16421	2410	<0.52	<3.23	7618	11833	146.5

Table 8. Elemental analysis (mg/Kg) for female gametophyte tissue of BC-1 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
BC-1	237.2	78.5	<0.37	2.2	21.2	129.2	28.3	12624	5774	<0.48	<3.00	4934	9503	240.8
BC-1	264.1	70.5	<0.38	2.8	19.5	131.8	23.3	12291	5883	<0.49	<3.06	4785	9343	198.6
BC-1	248.2	79.8	<0.38	2.3	19.8	129.4	31.2	12619	5775	<0.49	5.4	5128	9530	208.0
BC-1	257.3	77.3	<0.41	2.7	21.2	130.5	28.0	12636	5977	<0.52	<3.26	5199	9943	111.7
BC-1	205.8	65.6	<0.40	3.1	18.6	114.7	28.1	11907	5925	<0.51	<3.22	5050	8732	257.9
Mean	242.5	74.4		2.6	20.1	127.1	27.8	12415	5867			5019	9410	203.4
Std Er	10.2	2.7		0.2	0.5	3.1	1.3	143	41			73	197	25.3

Table 9. Elemental analysis (mg/Kg) for female gametophyte tissue from Union Camp 5-1036 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC5-1036	231.8	72.9	<0.40	<0.60	22.2	141.5	16.1	13448	5606	<0.52	<3.25	5455	9761	294.5
UC5-1036	241.5	88.4	<0.39	<0.58	22.2	151.9	20.8	13944	5600	<0.50	<3.14	5600	10024	402.2
UC5-1036	248.5	86.1	<0.39	<0.57	22.3	138.2	22.7	13218	5537	<0.50	<3.11	5278	9449	292.0
UC5-1036	211.7	79.2	<0.41	<0.60	21.2	141.2	22.9	13493	5444	<0.52	3.8	5477	9743	386.5
Mean	233.3	81.7			21.9	143.2	20.6	13525	5547			5453	9744	343.8
Std Er	8.0	3.5			0.3	3.0	1.6	152	38			66	118	29.4

Table 10. Elemental analysis (mg/Kg) for female gametophyte tissue of Westvaco 7-56 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
7-56	145.6	50.3	<0.34	<0.51	16.9	126.8	19.9	10913	4638	<0.44	3.9	4657	8150	253.5
7-56	100.0	51.1	<0.33	<0.48	15.4	120.5	16.1	10561	4451	<0.42	3.4	4570	7568	273.3
7-56	118.8	56.9	<0.33	<0.49	16.6	129.9	15.6	10936	4811	<0.42	3.3	4636	7811	284.3
Mean	121.4	52.7			16.3	125.7	17.2	10804	4633		3.5	4621	7843	270.3
Std Er	13.2	2.1			0.5	2.7	1.3	121	104		0.2	26	169	9.0

Table 11. Elemental analysis (mg/Kg) for female gametophyte tissue of Union Camp 10-14 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC10-14	210.5	80.3	<0.37	<0.54	17.4	157.8	17.7	11846	5472	<0.47	3.0	5381	8792	149.1
UC10-14	291.4	78.4	<0.39	<0.58	18.5	152.4	20.3	12116	5388	<0.50	4.3	5490	9412	150.6
Mean	251.0	79.4			17.9	155.1	19.0	11981	5430		3.6	5435	9102	149.9

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Table 12. Elemental analysis (mg/Kg) for female gametophyte tissue of Union Camp 10-1003 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC10-1003	287.0	66.4	<0.35	<0.55	21.3	192.7	11.6	12348	5465	<0.45	<2.83	5129	9460	310.7

Table 13. Ratio of average elemental concentrations in dried zygotic embryo vs. female gametophyte tissues.

Ratio	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
Zyg/FG	0.38	3.37	#####	0.80	1.37	0.95	0.29	1.34	0.46	#####	1.72	1.50	1.35	0.75

Table 14. . Elemental analysis (mg/Kg) for oven-dried somatic embryos of clone 195.

Somatics	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
SE	61.7	53	<0.72	<1.07	3.2	123.0	55	8157	3239	<0.93	829	4028	21703	485.6
SE	56.7	48	<0.44	<0.66	3.1	131.9	88	7939	2659	<0.57	1539	4153	23262	576.5
SE	42.6	46	<0.41	<0.61	2.2	116.3	91	6251	2241	<0.53	1576	3280	21645	590.0
Mean	53.7	49			2.8	123.7	78	7449	2713		1315	3820	22204	550.7
Std Er	5.7	2.0			0.3	4.5	12	602	290		243	273	529	32.8

Table 15. Comparison of elemental compositions for zygotic female gametophyte, zygotic embryo, and somatic embryo tissues with along with the ratio for each element found in somatic / zygotic embryos.

Tissue	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
Gametophyte	227	71	#####	3	20	149	19	12215	5388	#####	4	5132	9112	256
zygotics	81	231	#####	1.8	27.4	130	4.5	16246	2466	#####	6.9	7609	12075	182
Somatics	54	49			2.8	124	78	7449	2713		1315	3820	22204	551
Ratio Som/Zyg	0.66	0.21	#####	0	0.10	0.95	17.2	0.46	1.1	#####	191	0.50	1.8	3.0

Table 16. Elemental analysis (mg/Kg) for oven-dried somatic embryos of clone 333.

Medium	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
240 = 1x B, 1x Ca, 1x Fe	37.3	56.4	<2.10	<3.4	6.2	95.9	41.4	8989	3535	<5.0	1172	4369	27088	7395
705 = 1/2x B, 1/2 Ca, 1x Fe	28.3	36.5	<1.27	<2.1	3.6	83.6	<5.3	7594	3551	<3.0	1743	3976	26659	3338
751 = 3/4x B, 1/2x Ca, 1x Fe	51.4	59.8	<1.34	<2.2	6.3	82.7	20.4	9637	3632	<3.2	1088	4928	26310	4032
759 = 1/2x B, 1/2x Ca, 1.5x Fe	35.6	81.4	<1.39	<2.3	4.7	85.7	16.4	8576	3777	<3.3	1336	3999	29564	3604

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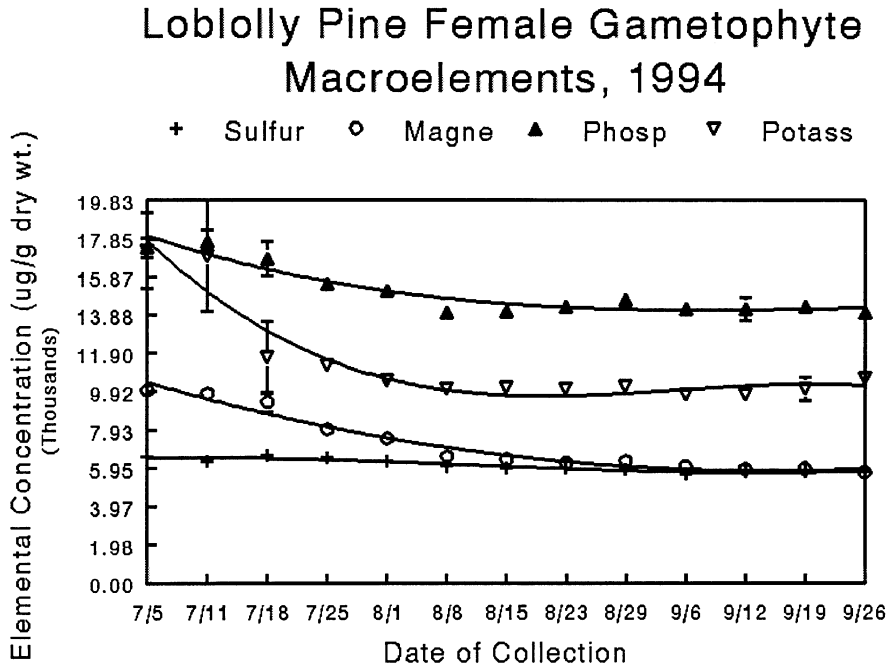


Figure 1. Elemental analysis (mg/Kg dry wt.) for female gametophyte tissue of Union Camp 10- 1036 seed collected in Summer 1994. Sulfur, magnesium, phosphorous, and potassium shown by date of collection of female gametophyte tissue.

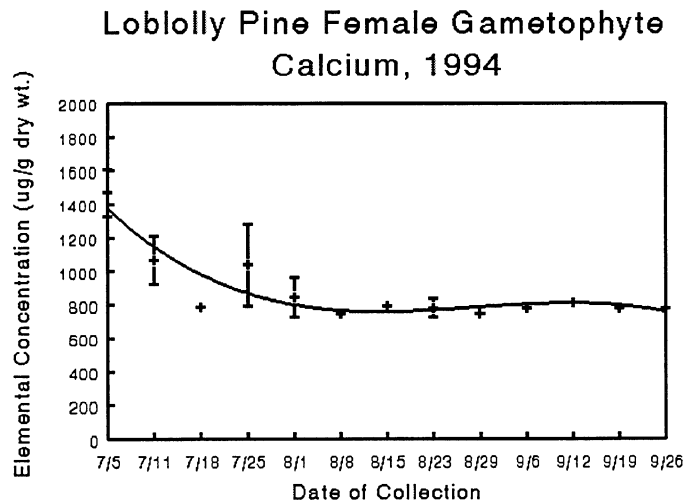


Figure 2. Elemental analysis (mg/Kg dry wt.) for female gametophyte tissue of Union Camp 10-1036 seed collected in Summer 1994. Calcium content shown by date of collection of female gametophyte tissue.

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Loblolly Pine Female Gametophyte Microelements, 1994

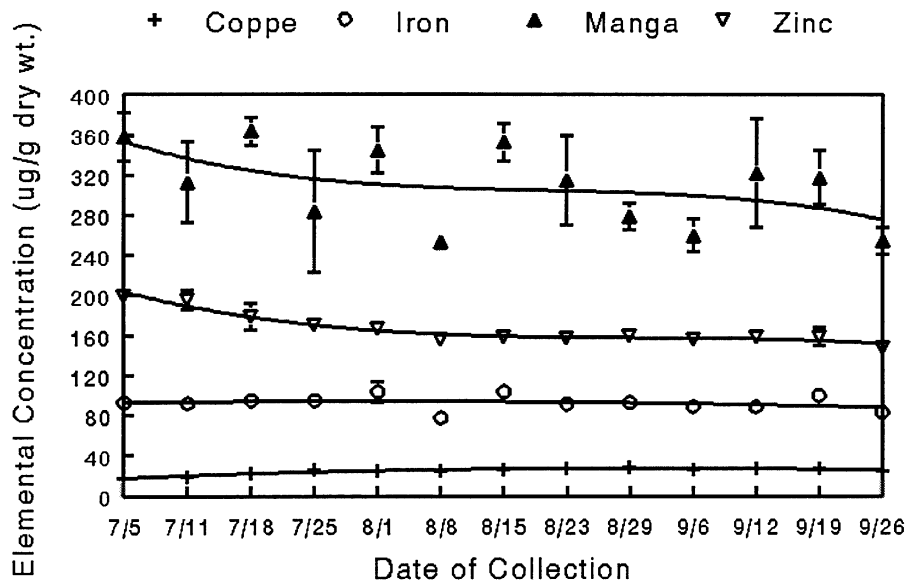


Figure 3. Elemental analysis (mg/Kg dry wt.) for female gametophyte tissue of Union Camp 10-1036 seed collected in Summer 1994. Copper, iron, manganese, and zinc content shown by date of collection of female gametophyte tissue.

Loblolly Pine Female Gametophyte Microelements, 1994

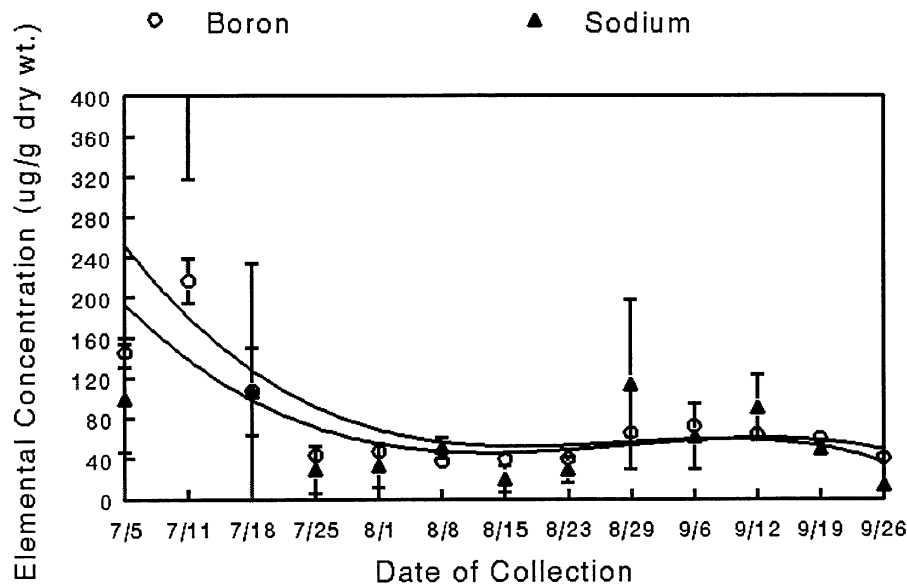


Figure 4. Elemental analysis (mg/Kg dry wt.) for female gametophyte tissue of Union Camp 10-1036 seed collected in Summer 1994. Boron and sodium content shown by date of collection of female gametophyte tissue.

Loblolly Pine Embryo Elemental Analy Macroelements

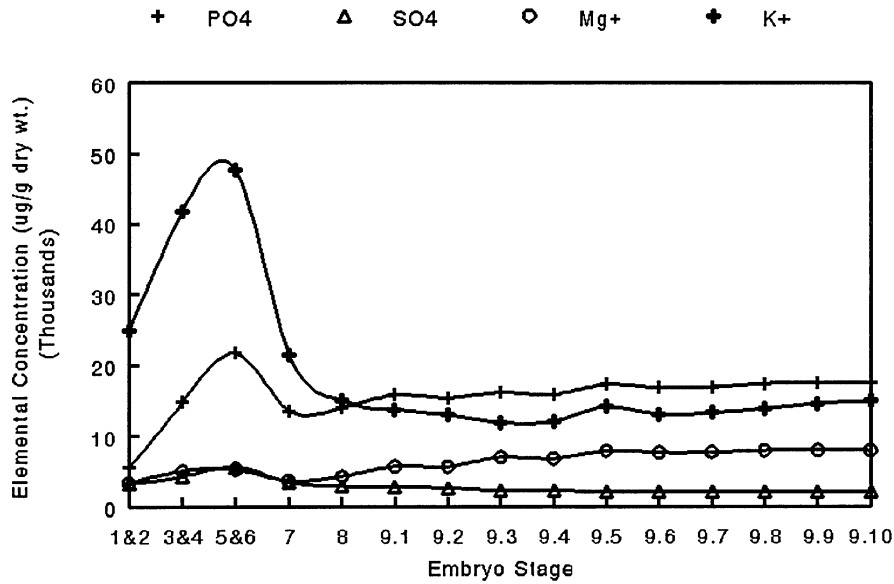


Figure 5. Elemental analysis (mg/Kg dry wt.) for zygotic embryo tissue of Union Camp 10-1036 seed collected in Summer 1994. Phosphorous, sulfate, magnesium, and potassium content shown by date of collection of embryo tissue.

Loblolly Pine Embryo Elemental Analy Calcium

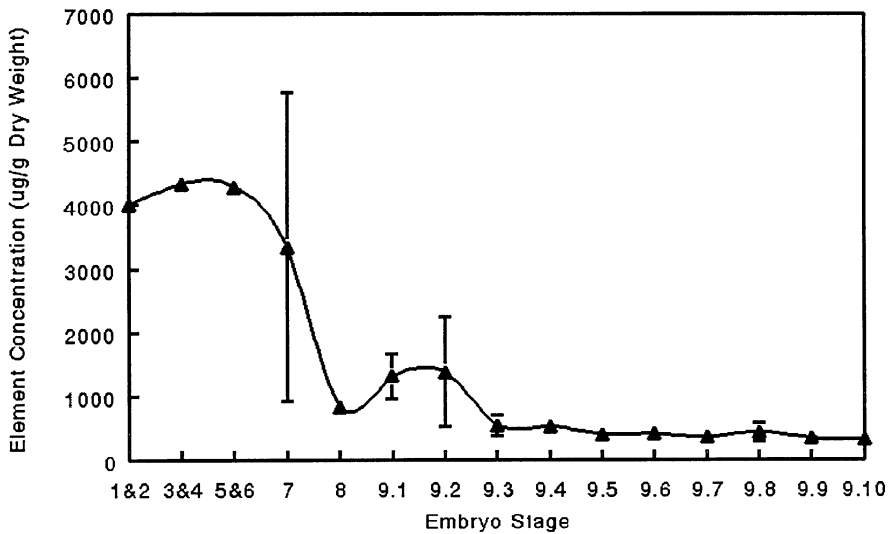


Figure 6. Elemental analysis (mg/Kg dry wt.) for zygotic embryo tissue of Union Camp 10-1036 seed collected in Summer 1994. Calcium content shown by date of collection of embryo tissue.

Loblolly Pine Embryo Elemental Analy Microelements

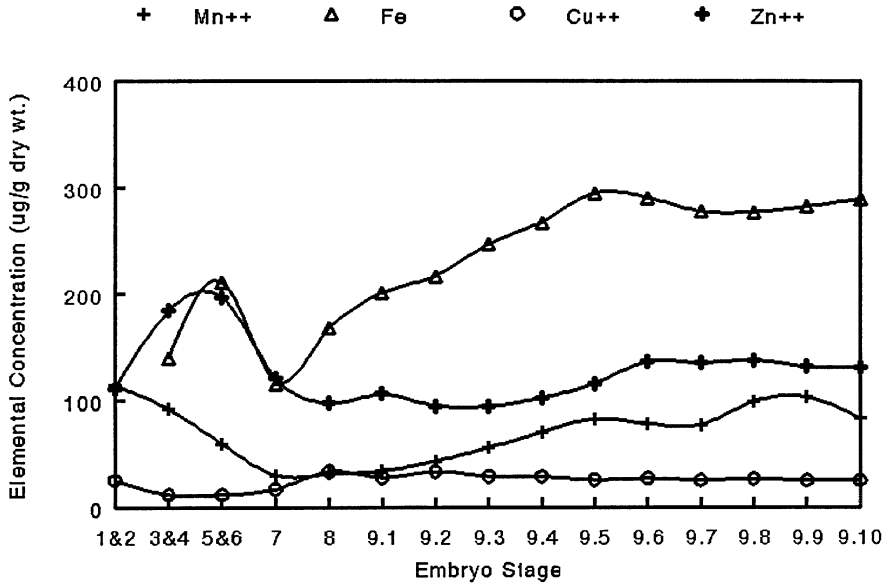


Figure 7. Elemental analysis (mg/Kg dry wt.) for zygotic embryo tissue of Union Camp 10-1036 seed collected in Summer 1994. Manganese, iron, copper, and zinc content shown by date of collection of embryo tissue.

Loblolly Pine Embryo Elemental Analy

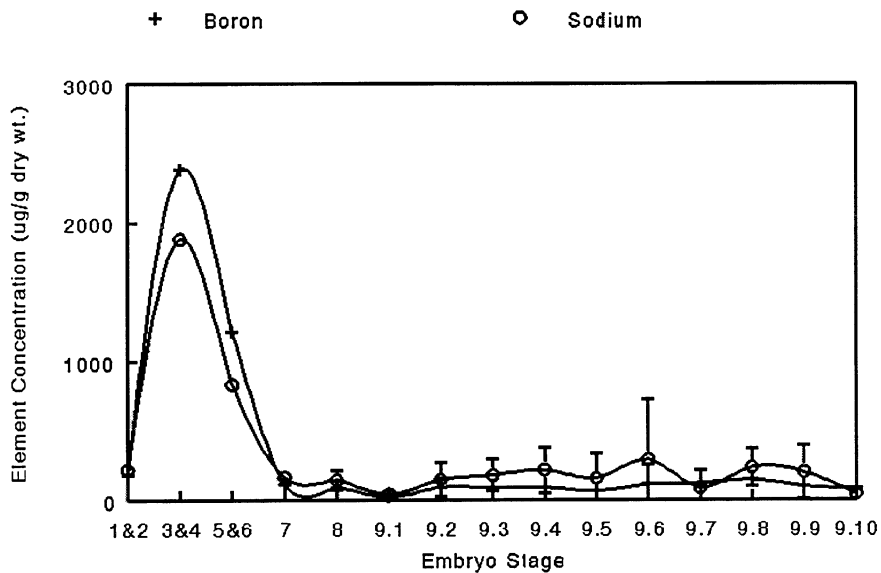


Figure 8. Elemental analysis (mg/Kg dry wt.) for zygotic embryo tissue of Union Camp 10-1036 seed collected in Summer 1994. Boron and sodium content shown by date of collection of embryo tissue.

F010: MASS CLONAL PROPAGATION OF SOFTWOODS
Functional Analysis of Zygotic Embryo Germination

Gary Peter
 Teresa Vales
 Chantal Murenzi
 Jennifer Langstraat
 Gerald Pullman

SUMMARY:

In previous studies at IPST comparisons of the fresh weight, dry weight, and patterns of gene expression between zygotic and somatic embryos, suggested that our somatic embryos develop to stages 8-9.1 In order to compare in a more functional way zygotic and somatic embryos, we tested the ability of immature zygotic embryos to germinate. The results show a parallel between the existence of a functional root meristem and germination. They also indicate that our somatic embryos develop only to stages 7-8. This suggests that early stages of somatic embryos development are occurring parallel to zygotic embryo development and that no major developmental step is missed. The results also suggests that our somatic embryos are not mature enough for normal germination, and that root growth appears to limit normal germination both in immature somatic and zygotic loblolly pine embryos.

INTRODUCTION:

In previous studies at IPST comparisons of the fresh weight, dry weight, and patterns of gene expression between zygotic and somatic embryos, suggested that our somatic embryos develop to stages 8-9.1. However, the somatic embryos that germinate do so in reverse sequence compared to zygotic embryos. Mature zygotic embryos germinate when the root emerges before or coincident with the shoot. In contrast when somatic embryos germinate, the cotyledons green first, the shoot emerges and then only much later if at all does the root appear. In order to compare the developmental potential zygotic and somatic embryos, we tested the ability of immature zygotic embryos to germinate. To determine when immature zygotic embryos acquire the capacity for normal germination we isolated embryos from stages 5-9.2 and placed them on germination media. From this functional study of zygotic germination capacity we can determine the developmental stage and the size and dry weights of immature zygotic embryos that are competent to germinate. This identifies a target for somatic embryo development. It also allows us to compare these results of immature zygotic embryos to the germination results from our somatic embryos that are also immature and obtain an estimate of somatic embryo quality. The results indicate that our somatic embryos develop to stages 7-8. The results also suggest that in our somatic embryos early stages of development probably occur normally and no major developmental step is missed.

EXPERIMENTAL APPROACH AND DESIGN:

Genotype UC 5-1036 was used this summer for mass embryo collections for initiation experiments. To perform germination tests, ovules were surface sterilized and the embryos were surgically isolated. Up to ten embryos from each stage were placed on one germination plate. This germination media did not contain exogenous hormones. Following our normal germination protocol, the embryos



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were then cultured in the dark for 7 days and subsequently shifted to continuous white light. After 6-7 weeks in the light the embryos were scored for the presence of roots and shoots under the dissecting microscope. An embryo was considered to have germinated when it contained both a root and a shoot. The hypocotyl and root lengths were measured when possible.

RESULTS:

Sterilization and Isolation Techniques Are Not Lethal.

Most embryos survived the sterilization and isolation procedure. Seeds were sterilized as for initiation experiments. Figure 1 shows that the most sensitive embryos were from stage 5, but still 80% of the embryos survived and continued to grow on hormone free media. In addition, the more mature the embryos were before isolation the greater their rate of survival, with 100% of the stage 9.2 embryos surviving (Figure 1).

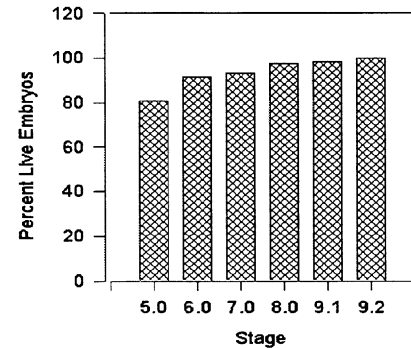


Figure 1

Cotyledon Development: The Number of Cultured Zygotic Embryos with Abnormal Cotyledons Decreases with Increasing Maturity

Zygotic embryos isolated at stage 5 showed the greatest percent (40) of embryos that formed abnormal cotyledons (Figure 2). The embryos that formed abnormal cotyledons were small and the axis was reduced. Cotyledons were abnormal either when the number of individual cotyledons was reduced or their morphology was altered. Mostly this was detected as fleshy and fused cotyledons (Figure 3, next page). By definition, stage 5 embryos have a shoot apical meristem or dome but no cotyledonary primordia are visible with the dissecting microscope. Thus, it was surprising that 60% of the zygotic embryos from this early stage of development when cultured on plates without hormone, developed normal cotyledons. One possible explanation for this is that microscopic cotyledonary primordia were present but not detectable with the dissecting microscope used. For stage 6 embryos, the earliest stage with cotyledonary primordia are visible, 84% formed normal cotyledons on our germination plates. About 85% of the stage 7-9.1 embryos formed normal cotyledons. Thus, once cotyledonary primordia become visible most are capable of normal growth and greening on our germination plates.

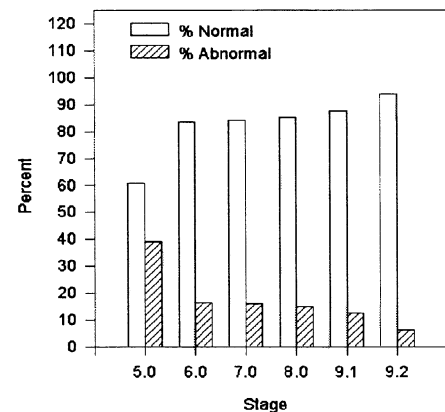


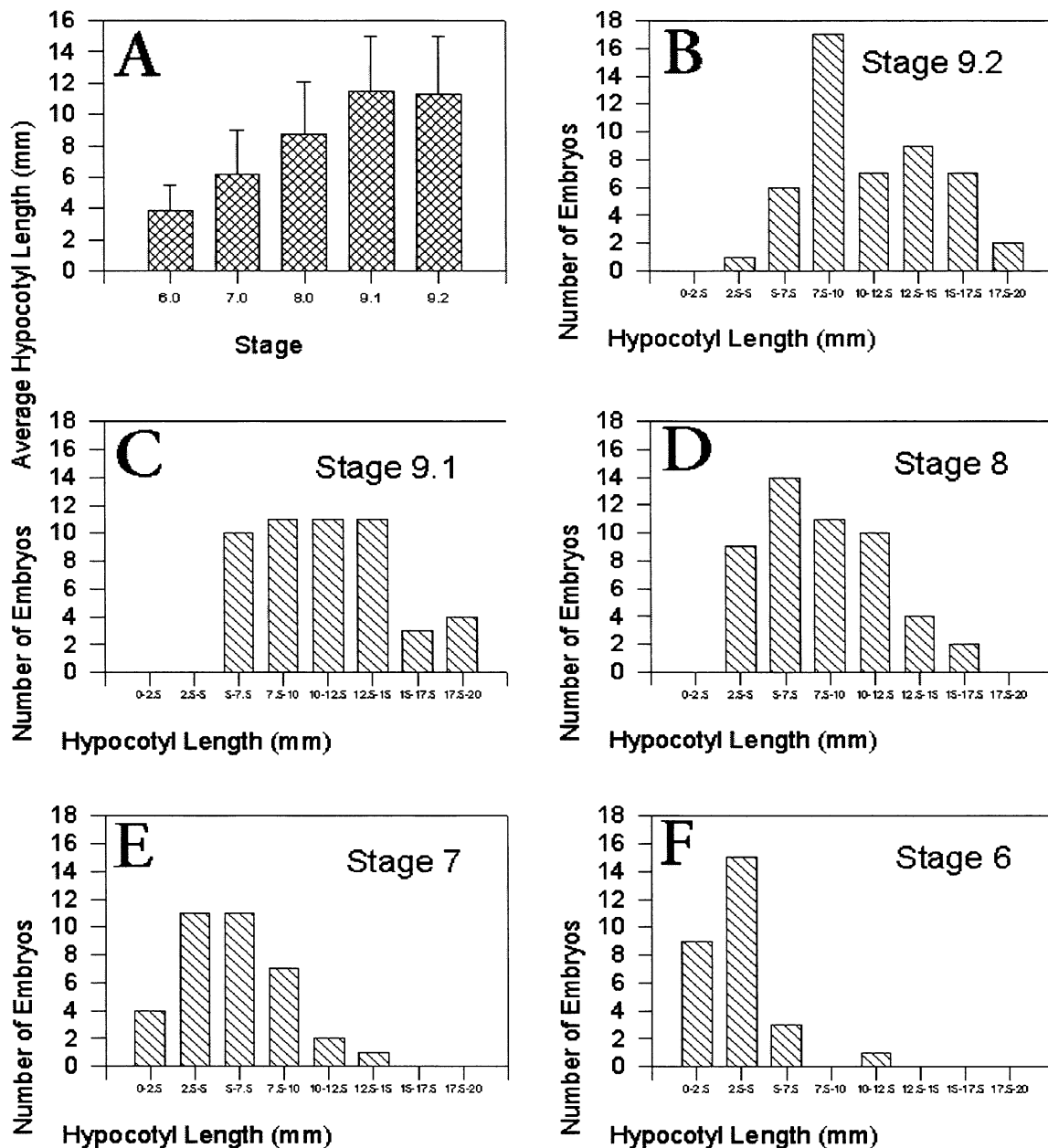
Figure 2

Hypocotyl Growth Increases with Maturity.



Zygotic embryos harvested later in development showed greater hypocotyl elongation than zygotic embryos harvested at earlier stages (Figure 4). For example, although shoots formed in ~50% of the stage 5 embryos (next page), the hypocotyls did not elongate. The hypocotyls began to elongate in stage 6 and 7 embryos (Figure 4F). From stages 6-9.2 the average hypocotyl lengths increased (Figure 4A) as did the distribution of longer hypocotyls (Figure 4B-E).

FIGURE 4



Development of a Functional Shoot Occurs as Early as Stage 5 and Possibly Before.

About 50% of the stage 5 embryos had formed a functional shoot meristem that developed after transfer to germination plates (Figure 5). This indicates that a functional shoot apical meristem is formed at or before stage 5. Our staging system defines a stage 5 embryo as one with an apical dome but without cotyledonary primordia; therefore shoot meristem development is probably complete when the dome is visible as evidenced by the autonomy of the shoot meristem to produce leaves without exogenous hormones. Why only ~50% of the embryos formed a functional shoot is unclear. However, 18% of the embryos formed abnormal cotyledons and none of these produced shoots. Of the stage 6 zygotic embryos, 63% formed functional shoots. This indicates that the apical meristem in at least half of the immature zygotic embryos does not require exogenous hormone treatment for it to be functional.

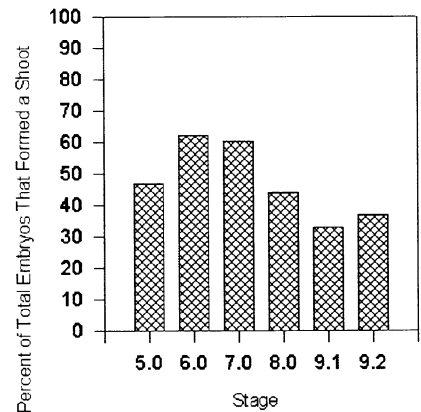


Figure 5

Development of a Functional Root Begins at Stage 7

No embryos from stage 5 and only 2 from stage 6 formed a root after harvest and transfer to germination plates. In contrast, at stage 7 ~18% of the zygotic embryos formed a functional root. The percent of embryos that formed a root increased with stage (Figure 6). Thus, functional roots are formed beginning at stage 7. Note this does not mean that the roots are not anatomically present at earlier stages. Roots may be present but have yet to acquire the ability or autonomy to germinate and grow.

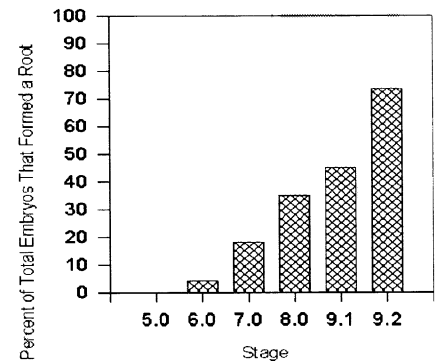


Figure 6

Root Growth Increases with Maturity

The average root length is relatively low for embryos harvested at stage 7 (Figure 7A, see next page). However, at stage 8-9.2 the average root length (Figure 7A) and the length distributions are similar for most roots (Figure 7B-D; see next page). One possible reason for the shorter roots at stage 7 is that the embryos took a longer time to germinate. Alternatively the roots might not be so competent for growth.

Zygotic Embryo Germination Begins at Stage 7 and Increases with Maturation.

The percent of embryos that germinate, i.e., form both a root and a shoot increases with stage (Figure 8). Figure 9 shows representative germinated embryos from each stage. Of the 48 stage 6 embryos only 1 germinated. Starting at stage 7 significant numbers of embryos germinated and the percent that germinated increased with stage. This confirms the straightforward notion that the stage to which a zygotic embryo develops is important for its ability to germinate. More importantly it gives us a target stage of maturity to aim for in developing maturation protocols.

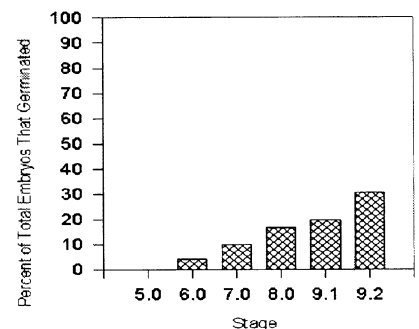
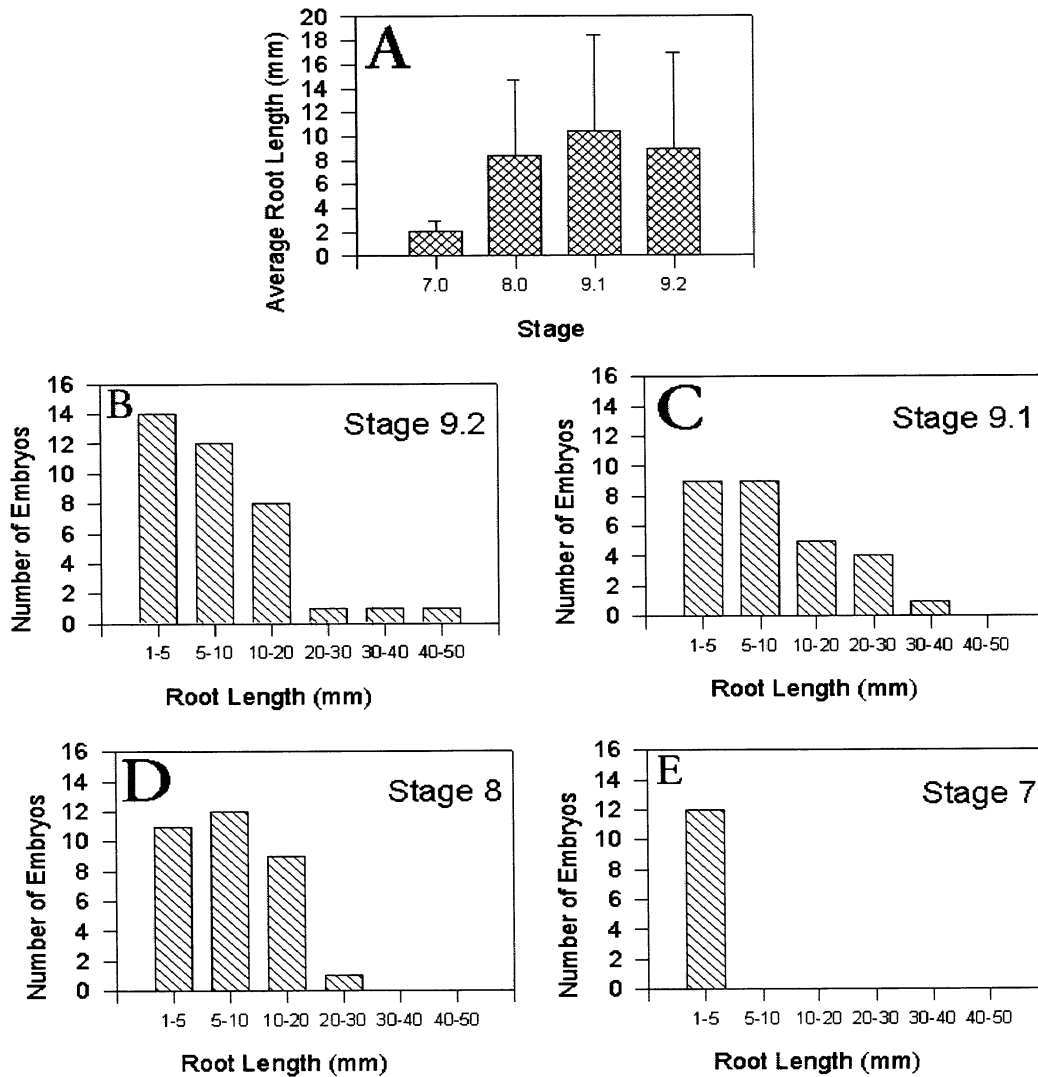


Figure 9 shows representative embryos that germinated (See next page).

FIGURE 7



Germination: A Fundamental Transition Occurs between Stages 8 and 9 of Zygotic Development.

At stage 9.1 and 9.2 more embryos that had a root only were observed than ones with a shoot only or that germinated (Table 1 and Figure 10). This is in striking contrast to embryos isolated at stages 6 and 7 in which only shoots grew and where very few roots were formed (Table 1 and Figure 10). Stage 8 had only slightly more embryos that formed only shoots versus ones that formed only roots or that germinated (Table 1 and Figure 10). This suggests that in embryos at stage 9.1 and beyond the root meristem is activated first upon germination. This is the natural sequence to mature zygotic embryo germination from seed. It also shows that a developmental transition occurs in the ability of the shoot to form first during zygotic embryo germination.

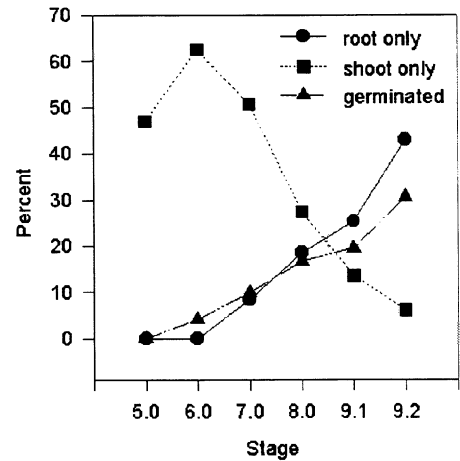


Figure 10

TABLE 1

Stage	Total # of Live Embryos	# of Embs with Roots Only	% of Embs with Roots Only	# of Embs with Shoots Only	% of Embs with Shoots Only	# of Embs Germinated	% of Embs Germinated
9.2	49	21	42.3	3	6.1	15	30.6
9.1	118	30	25.4	16	3.6	23	19.5
8	113	21	18.6	31	27.4	19	16.8
7	71	6	8.5	36	50.7	7	9.86
6	48	0	0	30	62.5	2	4.2
5	51	0	0	24	47	0	0

DISCUSSION:

A number of interesting observations were obtained from this study. One, root apical meristem autonomy develops after the shoot apical meristem autonomy during zygotic embryogenesis in loblolly pine. Two, when the root meristem does develop its autonomy it becomes the first organ to emerge from the embryo upon germination. Three, there is a parallel between the formation of a functional root and germination. Thus, root growth appears to limit normal germination both in immature somatic and zygotic loblolly pine embryos. Four, most of our somatic embryos form shoots first and then only a few much later form roots. This suggests that our somatic embryos are not mature enough for normal germination. Since we observe both shoot growth and hypocotyl growth in somatic embryos, these organs of the embryo develop normally. This suggests that early somatic embryo development, stages 1-6, are occurring normally and our block is in the later stages of embryo development. Autonomous root meristems are not forming in the somatic embryos and they are not going through the transition where the roots emerge first from the embryos.



F010 MASS CLONAL PROPAGATION OF SOFTWOODS
Update - Functional Analysis of Zygotic Embryo Germination

Gary Peter
Cielo Castillo
Teresa Vales
Jerry Pullman

Summary of Report from Fall 97 PAC:

In previous studies at IPST comparisons of the fresh weight, dry weight, and patterns of gene expression between zygotic and somatic embryos, suggested that our somatic embryos develop to stages 8-9.1 In order to compare in a more functional way zygotic and somatic embryos, we tested the ability of immature zygotic embryos to germinate. The results show a parallel between the existence of a functional root meristem and germination. They also indicate that our somatic embryos develop only to stages 7-8. This suggests that early stages of somatic embryos development are occurring parallel to zygotic embryo development and that no major developmental step is missed. The results also suggests that our somatic embryos are not mature enough for normal germination, and that root growth appears to limit normal germination both in immature somatic and zygotic loblolly pine embryos.

Conclusions from Experiment:

1. Root apical meristem autonomy develops after the shoot apical meristem autonomy during zygotic embryogenesis in loblolly pine.
2. When the root meristem does develop its autonomy it becomes the first organ to emerge from the embryo upon germination.
3. There is a parallel between the formation of a functional root and germination. Thus, root growth appears to limit normal germination both in immature somatic and zygotic loblolly pine embryos. Most of our somatic embryos form shoots first and then only a few much later form roots. This suggests that our somatic embryos are not mature enough for normal germination.
4. Since we observe both shoot growth and hypocotyl growth in somatic embryos, these organs of the embryo develop normally.
5. This suggests that early somatic embryo development, stages 1-6, are occurring normally and our block is in the later stages of embryo development. Autonomous root meristems are not forming in the somatic embryos and they are not going through the transition were the roots emerge first from the embryos.

Questions Left to be Answered From Experiment:

1. When during zygotic embryo development does the root apical meristem form? Is this before root meristem autonomy is obtained?
2. Do most somatic embryos develop a histologically identifiable root apical meristem?



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Follow up Experiments:

When during zygotic embryo development does the root apical meristem form?

Background: The results from our functional studies of the *Pinus taeda* zygotic embryo germination showed that the competency of the shoot to emerge was obtained by stage 5; whereas, the competency of the root to emerge from the zygote occurred only by stage 7 and increased gradually in the later stages. When the root apical meristem gains the autonomy to emerge from the embryo correlated highly with overall zygotic embryo germination; therefore, root emergence limited normal germination. To determine whether the formation of the root apical meristem itself or the acquisition of root meristem autonomy is what limited the roots ability to emerge from isolated early stage embryos we needed to determine when the root meristem is first visible in *Pinus taeda*. Previous studies with *Pinus strobus* showed that during zygotic embryo development the root apical meristem is clearly visible by our stage 4 when the shoot apical meristem is detected. (A. Spurr (1949). Histogenesis and Organization of the Embryo in *Pinus strobus* L. *Am. J. Botany* 36 (9): 629-641). Thus, if this is also true for *Pinus taeda* then it suggests that a change in competency of the embryonic root to germinate occurs later in development and may limit normal germination.

Methods: Intact ovules were collected during each week of zygotic development last summer. These ovules were fixed with 4% glutaraldehyde and embedded into paraffin wax for future sectioning. Ovules that were thought to contain embryos ranging from stage 4-9.1 were sectioned, mounted, deparaffinized, hydrated and stained with safranin-fast green.

Results: The results of these sectioning experiments will be presented at the PAC meeting. It is expected that like *Pinus strobus* that root apical meristems develop around the time the shoot apical meristem does during zygotic embryogenesis of loblolly pine. If this result is observed then it shows that the autonomy of the root to germinate before the shoot is acquired late in development.

Do most somatic embryos develop a histologically identifiable root apical meristem?

Background: In general, our somatic embryos germinate like young zygotic embryos of - stage 7: a shoot emerges first and then only much later if at all a root emerges. To confirm our initial conclusion that the early stages of somatic embryo development occur normally, we wanted to determine whether a root apical meristem is formed during somatic embryo development. If a root apical meristem is present in most embryos this suggests that our somatic embryos do not reach the stage of the development where root autonomy for germination is attained.

Methods: Full size somatic embryos (3 months old) were fixed with 4% glutaraldehyde and embedded into paraffin wax, were sectioned, mounted, deparaffinized, hydrated and stained with safranin-fast green.

Results: It appears that many of the somatic embryos do form a histologically identifiable root apical meristem. The sectioning results will be presented at the PAC meeting.



Mass Clonal Propagation of Improved Conifers (Project F010)

The Role of the Suspensor in Early Embryo Development.

I. Differential gene expression in the suspensor of zygotic embryos

John MacKay
Heidi Schindler
Jerry Pullman

SUMMARY

The goal of this research is to investigate suspensor biology in zygotic embryos and to use the information learned from zygotic embryos to assess how we could improve the quality of somatic embryos by adapting tissue culture conditions. This specific report describes experiments toward the identification of genes that are differentially expressed in the suspensor of immature zygotic embryos. The purpose of these gene isolation experiments is to gain insights into suspensor biology and to potentially identify markers of suspensor development. Genes that are specifically or more abundantly expressed in the suspensor are being isolated by the construction of complimentary DNA (cDNA) libraries that are enriched for such genes by using a method called subtractive hybridization. Preliminary results show that numerous high quality cDNAs are thus obtained and many of these cDNAs are confirmed to be more abundant in the suspensor. Although only a few of cDNAs have been sequenced, several of them gave strong matches with seed storage proteins.

INTRODUCTION

The suspensor tissue is found at the base of the embryo and supports the embryo during their early to middle stages of development. In early stage zygotic embryos of loblolly pine (stages 1 to 4), the suspensor comprises most of the embryo's mass (Figure 1). We hypothesize that the suspensor plays an important, yet poorly understood, role in embryogenesis of loblolly pine both for zygotic and somatic embryos. Therefore, the formation and early development of **somatic embryos** should require suspensor functions, which may be uncovered by investigating the role of the suspensor in **zygotic embryos**. A better understanding of the role and development of the zygotic suspensor could allow us to better assess the effect of culture conditions and, make improvements to the initiation and multiplication phases of somatic embryogenesis. A better understanding of embryo development at early stages may also be essential to improving the quality of late stage embryos.

In angiosperm plants, the suspensor is required for early embryo development and, three different roles have been attributed to the suspensor (Cionini, 1987). First, the suspensor is considered to have a role of mechanical support because it holds the embryo in place and pushes it into the corrosion cavity of the seed. Second, the suspensor plays a nutritional role, such as the synthesis of storage proteins which can be mobilized to sustain embryo growth. Third, the suspensor has been implicated as potentially regulating embryo growth and development because it synthesizes growth regulators, namely gib-

berellins. These roles highlight the importance of the suspensor in angiosperms but have only been partially verified in conifers.

As a first step toward assessing the biological role of the suspensor in pine, we have undertaken experiments to identify genes that may be differentially expressed in the suspensor of immature zygotic embryos. We expect that this approach will yield valuable information on specific functions of the suspensor. Currently available molecular biology techniques which utilize PCR to isolate genes of interest should allow us to overcome the limitations imposed by the small size of immature embryos and the difficulty of dissecting tissues from a large number of them.

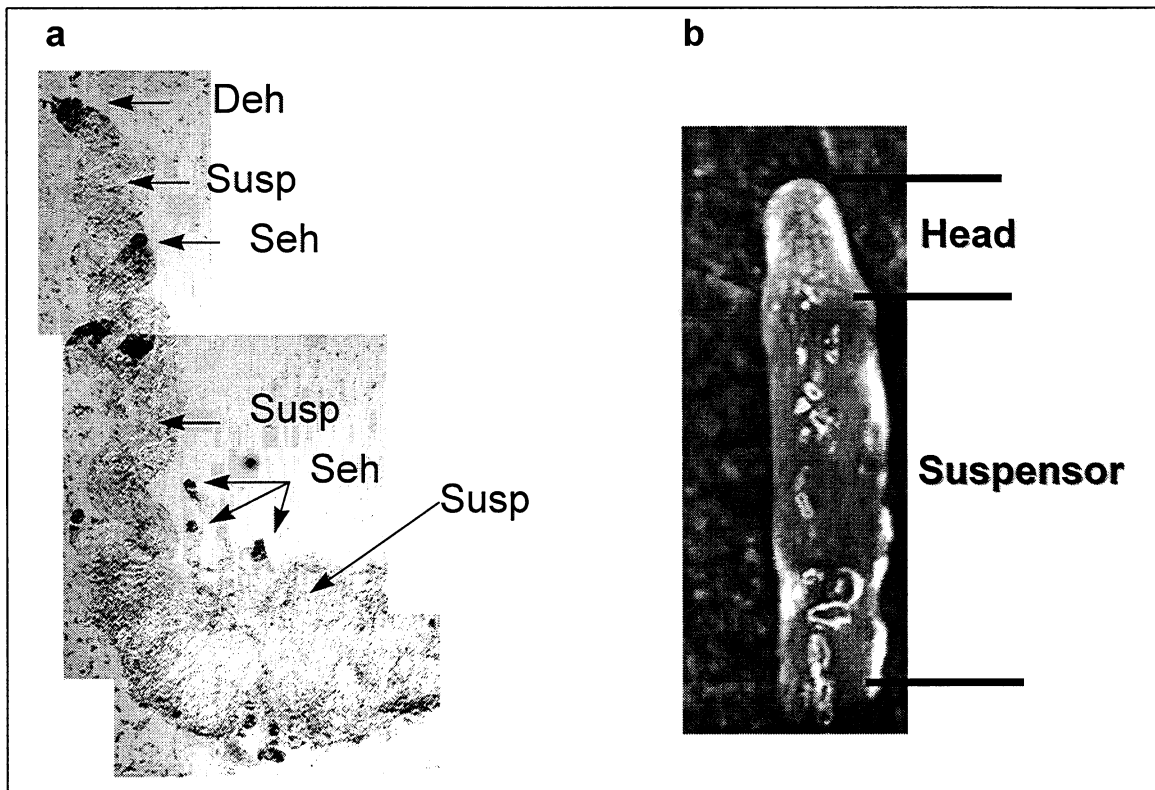


Figure 1. The suspensor of immature of loblolly pine zygotic embryos. a. Stage 2 embryo stained to reveal the dominant and subordinate embryos supported by the suspensor mass, Deh: Dominant embryo head; Seh: Subordinate embryo head; Susp: Suspensor; b. Unstained stage 3 embryo.

RESULTS

Isolation of tissues and RNA preparation.

Embryo head, suspensor and megagametophyte tissues were isolated from immature zygotic loblolly pine embryos, collected in July. Tissue samples were collected from immature embryos of developmental stages 2, 3, 4 and 5 and kept separate and, samples from different mother-trees (genotypes 5-1036, 5-1507) were also kept separate. Poly A⁺ RNA (messenger RNA) was isolated using oligo dT coated magnetic beads

(Dynabeads, Dynal, Inc.) following Rosok *et al.* (1996). RNA was isolated from embryo head and suspensor tissues of 36 stage 3 embryos, of 24 stage 4 embryos and from five megagametophytes for each stage.

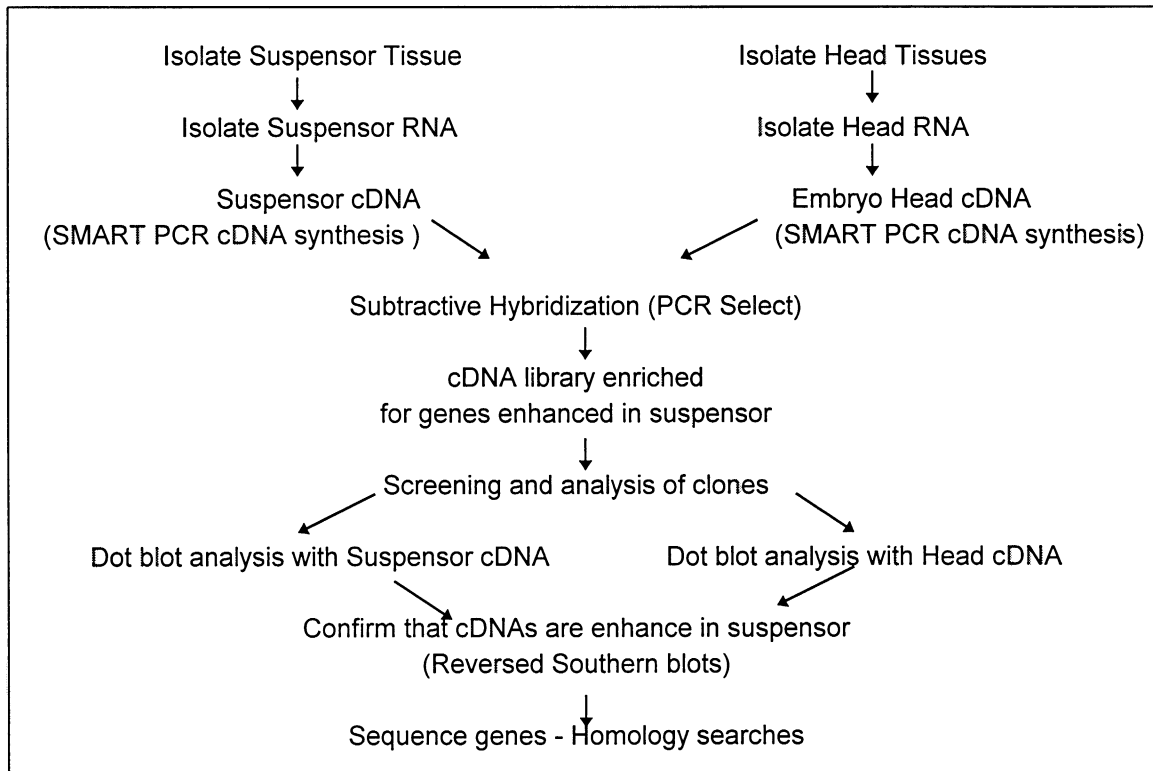


Figure 2. Flow chart of the isolation and screening of differentially expressed suspensor cDNAs.

Construction of suspensor enriched cDNA libraries by subtractive hybridization.

We generated two cDNA libraries (one from stage 3 embryos and one from stage 4) that are enriched for gene sequences which are suspensor specific or are more abundant in the suspensor. Figure 2 presents a flow chart of the steps involved in the preparation and screening of these libraries. The libraries were obtained by subtractive hybridization of embryo head cDNAs from embryo suspensor cDNAs, resulting in a population of cDNAs enriched for genes uniquely expressed in the suspensor or more abundantly expressed in the suspensor. Due to the very small amounts of starting RNA for these experiments, we utilized PCR based methods to generate the original cDNA pools (SMART PCR cDNA synthesis kit, Clontech) and to carry out the subtractive hybridization (PCR Select kit, Clontech). The resulting cDNAs were cloned in bulk into a plasmid vector (pT7Blue-III) and, we screened 357 plasmids each potentially containing a cDNA clone (194 from the stage 3 library, 163 from the stage 4 library). Colony PCR was used for this screening and showed that 89% of the plasmids contained a cloned DNA fragment, and these ranged from 300 bp to 1500 bp in size (Figure 3a). The products from the colony PCR screening were digested with the restriction enzyme HhaI to obtain preliminary information on the DNA sequence (Figure 3b). The restriction enzyme analysis indicated

that the cDNA fragments were very diverse, less than 20% of them appeared similar to another clone in the study.

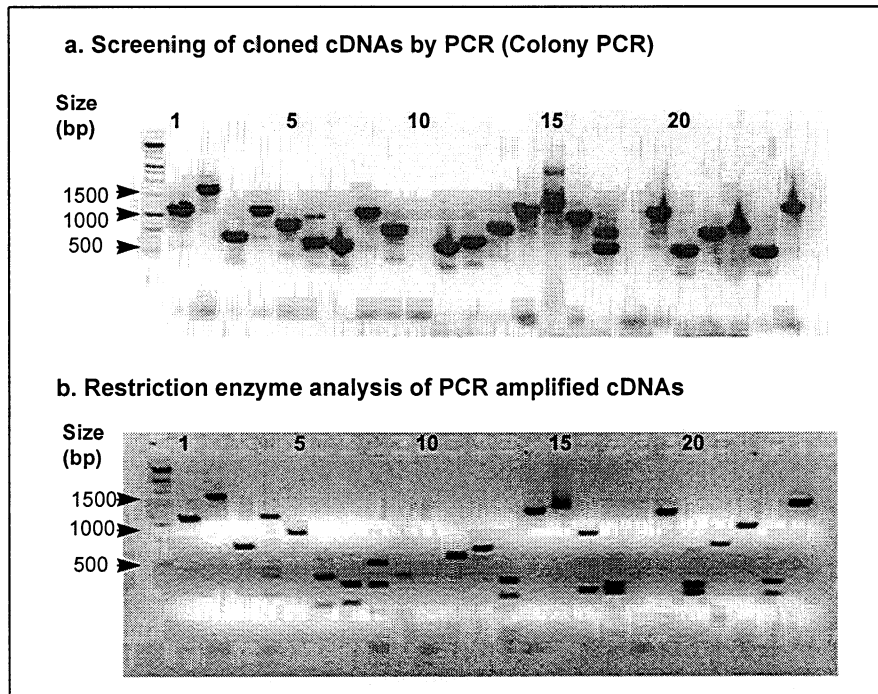
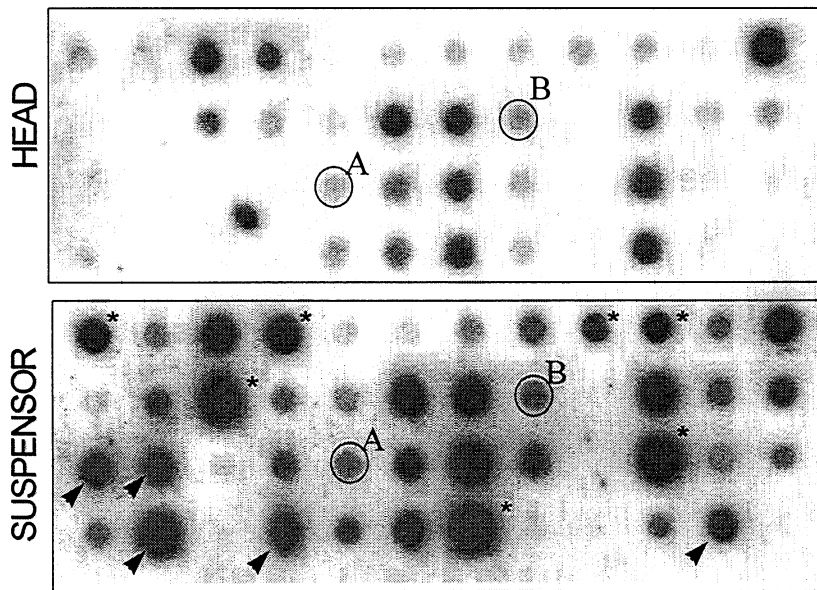


Figure 3. Screening of cDNA fragments cloned after subtractive hybridization of suspensor and embryo head cDNAs **a.** Electrophoresis of PCR analysis to verify the presence and size of cDNA inserts, each lane is an individual randomly selected clone. **b.** Electrophoresis of the same PCR amplified cDNAs, digested with the restriction enzyme *Hha*I, resulting in one to several DNA fragments. Molecular size standards are on the left.

Identification of abundantly expressed suspensor genes by blot analysis

We have begun to verify the tissue specificity of expression of the cDNA sequences of our subtracted libraries, by using reversed Southern blot analysis. A subset of 240 cDNA clones were blotted onto duplicate membranes and probed with cDNAs (non subtracted) from embryo head and embryo suspensor separately (Figure 4). The results from each probe were compared to determine the relative abundance of each cDNA in the suspensor and in the embryo head. A ribosomal RNA gene was included on the dot blots as a control to facilitate blot to blot comparison of different probes. Currently this comparison is strictly qualitative however quantitative analyses are planned for future experiments. We have identify 53 cloned cDNAs that were more abundant in the suspensor. Future experiments are needed to analyze more clones, verify repeatability using other genotypes and include comparison to the megagametophyte. In addition, this method is not suitable for detecting low abundance and rare messages, therefore other methods such as RNase protection assays should be used to attempt to detect suspensor specific genes that are less abundant, which is often the case with genes which encode regulatory proteins.



➤ Suspensor Specific * More abundant in Suspensor

Figure 4. Identification of suspensor abundant and putative suspensor specific cDNAs by reversed Southern Dot Blot. The top and bottom panels are identical replicates of one another and were probed either with labeled embryo head cDNA (top) or with embryo suspensor cDNA (bottom). A ribosomal RNA gene was included as two control dots (circled and marked A and B).

Table 1. Summary of progress toward isolation of suspensor specific genes.

	Number
Clones isolated and screened	357
Clones tested by reversed Southern blot	240
Clones with of higher abundance in suspensor	53
Clones sequenced	9
Clones with homology to known proteins	6

Sequence analysis of suspensor cDNAs

The DNA sequence was determined for a subset of 9 suspensor abundant clones and homology searches were carried out against public gene databases. A majority of these 9 clones gave matches with genes encoding proteins of known function. Five of the 9 showed homology with seed storage proteins, one matched with a cysteine proteinase inhibitor, two others cDNA appeared to be related to a nematode protein of unknown function. Only one out of 9 gave no hits. More cDNAs are being sequenced.

Table 2 Sequence homology search for some suspensor abundant cDNAs isolated by subtractive hybridization.

cDNA clone	Stage	cDNA size(bp)	Highest match in database	Probability score
1-3*	3	800	Albumin 3 (seed storage protein), white pine	10^{-110}
201	3	1300	Globulin (seed storage protein), white pine	10^{-35}
207	3	1250	Globulin (seed storage protein), white pine	10^{-106}
220	3	700	Albumin 1 (seed storage protein), white pine	10^{-86}
228	3	1300	Vicilin (seed storage protein), white spruce	10^{-131}
238	3	1300	Protein of unknown function, nematode	2×10^{-20}
419	4	1100	same as 238	10^{-29}
504	4	900	Cysteine proteinase inhibitor	10^{-21}
514	4	900	No matches	

*cDNA clone 1-3 was suspensor abundant but not isolated by subtractive hybridization

DISCUSSION AND CONCLUSION.

The approach described in this report relies on PCR for the synthesis of cDNAs and for subtractive hybridization. PCR has the advantage of requiring very little starting material and without such methods it would not be possible to effectively study differential gene expression in pine embryos. PCR may have significant drawbacks however. Experiments must be carefully controlled to avoid generating artifacts, isolating contaminants or dramatically distorting the composition of the RNA transcript pool. We are encouraged by the fact that so far, a high proportion of the clones isolated seem to be of good quality and give strong matches with genes already in the database. Several of the genes we have isolated and sequenced so far, appear to encode seed storage proteins. Recently it was reported that the suspensor of *Vicia faba* (field bean) has a significant but transient accumulation of storage proteins (Panitz et al., 1995) which most likely contributes to the nutritional role of the suspensor. The accumulation of storage protein has also been observed in immature zygotic embryos of spruce but no report details the putative localization of these proteins (Flinn et al., 1993). Our findings are still preliminary and experiments to verify the enhanced expression of the our cDNAs in other loblolly pine genotypes and, using other methods are still needed. If confirmed, our results would indicate that suspensor functions that have been characterized in angiosperms could be directly tested in pine. The type of study we have undertaken also has the potential to uncover other features that may be unique to the suspensor of pine and other gymnosperms.

An important issue that needs to be addressed is how the isolation and characterization of these genes can provide useful information for improving somatic embryogenesis methods to give embryos of better quality. From the knowledge gained by careful studies of gene expression, molecular and biochemical markers could be developed to assess embryo (suspensor) development and growth in the early stages of

somatic embryogenesis. For example, in this study of suspensor gene expression, we have identified several cDNAs encoding putative storage proteins. Although this finding does not establish the relative abundance of these proteins in the suspensor, it suggests that further studies could be directed to the synthesis of some of these storage proteins by suspensor cells in somatic embryogenesis. Selected storage proteins or their genes could be used as markers to explore how tissue culture treatments may effect their synthesis and mobilization. Markers of suspensor development and function may also be useful to follow the evolution and fate of different cell types in somatic cultures, during culture cycling or culture decline for example. To develop useful markers, it will be necessary to clearly show the temporal and spatial specificity of gene expression of selected genes.

Finally, the development of methods that utilize large arrays of genes to survey gene expression could be used to rapidly collect data on the effect of specific tissue culture treatments. The utility of gene arrays will depend in part on our ability to assay gene expression quantitatively. We have begun using a ribosomal RNA gene as a control for the purpose of comparing signal intensity on replicate blots probed with different probes, however other potential controls for quantitative comparisons should also be tested. Through these experiments and together with other projects in the Forest Biology Group, we are developing molecular analysis tools for the improvement of somatic embryo quality.

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**EXTERNALLY FUNDED RESEARCH
in 1997-1998
SUPPORTING F010**

**John Cairney
Gerald Pullman
Gary Peter
Ranjan Perrera
Nanfei Xu
Byron Waldrop
Michael Sullivan
Steve Van Winkle
Vincent Ciavatta**

March 26-27, 1998

Gene expression in loblolly pine embryos: cDNA cloning, high density array and gene expression detection.

Nanfei Xu, Heidi Schindler, Gerald Pullman and John Cairney

Summary:

The development of a embryo is the result of the expression of many genes in a temporally and spatially regulated fashion. The difference in the quality between somatic and zygotic embryos lies in the difference in their gene expression. The detection of gene expression in both kinds of embryos at different developmental stages should be crucial in improving the quality of somatic embryos. To this end, we planed to clone several hundred cDNAs that represent genes differentially expressed at different stages of zygotic embryo development. This collection of cDNAs can then be used as probes to detect the expression of corresponding genes in the embryos. To date, we have cloned 417 cDNAs, made high density cDNA arrays, and developed highly sensitive and reproducible method for detecting the expression of these genes.

Introduction:

High quality somatic embryos are valuable resources for quick propagation of desirable forest tree species. Although somatic embryos have been produced in several forest species including loblolly pine, the quality of these embryos needs to be improved for use at a commercial scale. The quality of the somatic embryos is usually measured by their resemblance to their zygotic counterparts, both in germinability and morphology. Currently, researches are focusing on the morphological, physiological and biochemical improvement of the somatic embryos through manipulations of the culture conditions. The changes in morphology, and physiology of the embryos in any experimental treatment is usually observed long after the treatment was applied. Also, the morphological and physiological effects are the combined results of many genes expressed early during the culture. It would be more informative and precise if the effects of any treatment can be dissected into individual changes in gene expression and into different time points along stages of development. Numerous genes are expressed during embryogenesis; many of them are called "house-keeping" genes. It is currently impractical to characterize all these expressed genes. Our proposed approach is to characterize a set of genes that are only expressed during specific stages of embryogenesis. The first step in this approach is to clone these genes. This report lists all the genes we have cloned and briefly describes the high density cDNA array hybridization technique we have developed.

Results and Discussion:

Cloning:

Cloning of cDNAs from differential display bands has been described in previous PAC reports. Recent progress is high lighted here. Since the last PAC meeting we have cloned 223 more cDNAs from over 300 differential display bands from zygotic embryos. This brings the total cDNAs cloned from zygotic embryos to 321 and the total cDNAs from both somatic and zygotic embryos to 417. We have sequenced all the 223 new cDNAs from zygotic embryos and 15 cDNAs from somatic embryos. This brings total sequenced cDNAs to 417. Sequence data has been processed and homology searched among all the clones and in the GenBank.



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According to current available data, the 417 cloned sequences belong to 345 groups; 227 of them are novel and 118 have various degree of homology to GenBank sequences. This work is summarized in Table 1 and the list of GenBank homology is in Table 2.

Table 1. Statistics of Cloned cDNAs

	Somatic Embryos	Zygotic Embryos	Total
cDNAs cloned	96	321	417
Clones sequenced	96	321	417
Unique groups	86	259	345
Novel groups	68	159	227
GenBank hits	18	100	118
Example hits	DC3 promoter GTP binding binding factor, proteins, Serine Kinase GA related protein,		



Table 2: Homology of cloned loblolly pine cDNAs to GenBank sequences.

* pH:, pM:, pL:, homology of translated peptide at high level (BLAST score >200), middle level (BLAST score between 150 and 200) and low level (BLAST score between 80-149).

dH:, dM:, dL:, homology of DNA sequence at high level (BLAST score >400), middle level (BLAST score between 300 and 399) and low level (BLAST score between 200-299).

LPS001	Novel	LPS043	Novel
LPS002	Novel	LPS044	Novel
LPS003	Novel	LPS045	Novel
LPS004	Novel	LPS046	Novel
LPS005	Novel	LPS047	Novel
		LPS048	Novel
		LPS049	dM:N. tabacum rab7b mRNA
		LPS050	Novel
		LPS051	Novel
		LPS052	Novel
LPS006	Novel	LPS053	dH:tetraphosphate hydrolase, dM:N. tabacum rab7b mRNA
LPS007	Novel		
LPS008	Novel	LPS054	Novel
LPS009	Novel	LPS055	dL:a human cDNA clone
LPS010	Novel	LPS056	Novel
LPS011	pL:GTL1 transcription factor, dH:Oxygen evolving complex protein	LPS057	Novel
LPS012	Novel	LPS058	Novel
LPS013	Novel	LPS059	dL:loblolly pine cDNA clone 2897s
LPS014	Novel	LPS060	dH:Tropaeolum majus XET1 cDNA clone
LPS015	Novel	LPS061	Novel
LPS016	Novel	LPS062	Novel
LPS017	Novel	LPS063	Novel
LPS018	Novel	LPS064	Novel
LPS019	Novel	LPS065	dL:mouse embryonic ectoderm development protein mRNA
LPS020	Novel		
LPS021	Novel	LPS066	Novel
LPS022	Novel	LPS067	pM:Arabidopsis zeta crystallin
LPS023	Novel	LPS068	Novel
LPS024	pL:snRNA associated protein	LPS069	Novel
LPS025	Novel	LPS070	Novel
LPS026	Novel	LPS071	Novel
LPS027	Novel	LPS072	Novel
LPS028	Novel	LPS073	dH:Arabidopsis expressed tag
LPS029	Novel	LPS074	Novel
LPS030	Novel	LPS075	dH:L. esculentum tXET-b1 mRNA
LPS031	Novel	LPS076	Novel
LPS032	Novel	LPS077	Novel
LPS033	Novel	LPS078	pH:rat 60S ribosomal protein L35
LPS034	Novel	LPS079	Novel
LPS035	Novel	LPS080	pL:Xenopus laevis Etr-3, dL:loblolly pine cDNA clone 2345e
LPS036	Novel		
LPS037	Novel	LPS081	Novel
LPS038	pH:Chlorella kessleri EF-2	LPS082	Novel
LPS039	pH:Chlorella kessleri EF-2	LPS083	Novel
LPS040	pM:Dengen virus nonstructural protein 1	LPS084	Novel
LPS041	Novel	LPS085	dH:P. amygdalus alfa-tublin mRNA
LPS042	Novel	LPS086	Novel



LPS087	Novel	LPZ053	dH:rice cDNA S3941_1A
LPS088	Novel	LPZ054	Novel
LPS089	Novel	LPZ055	Novel
LPS090	Novel	LPZ056	Novel
LPS091	Novel	LPZ057	pL:C. elegans ZK792.1 sequence
LPS092	dM:Arabidopsis cDNA clone AR192	LPZ058	pL:C. elegans ZK792.1 sequence
LPS093	dH:human serine kinase mRNA	LPZ059	pL:peptide transposon BU-24, dL:lily del transposon
LPS094	pH:H. annuus DC3 promoter binding factor	LPZ060	dL:black pine chloroplast DNA
LPS095	Novel	LPZ061	Novel
LPS096	Novel	LPZ062	Novel
LPZ001	Novel	LPZ063	Novel
LPZ002	Novel	LPZ064	Novel
LPZ003	Novel	LPZ065	dH:rice Rr15 mRNA for 5S ribosomal RNA
LPZ004	Novel	LPZ066	pL, dL:acyl-CoA-binding protein
LPZ005	Novel	LPZ067	Novel
LPZ006	Novel	LPZ068	dL:rice Rr15 mRNA for 5S ribosomal RNA
LPZ007	pL:Arabidopsis receptor protein kinase	LPZ069	Novel
LPZ008	dH:Lily mRNA from flowering buds	LPZ070	Novel
LPZ009	Novel	LPZ071	Novel
LPZ010	dL:human aorta cDNA	LPZ072	Novel
LPZ011	Novel	LPZ073	Novel
LPZ012	Novel	LPZ074	Novel
LPZ013	Novel	LPZ075	Novel
LPZ014	Novel	LPZ076	Novel
LPZ015	Novel	LPZ077	Novel
LPZ016	pL:Arabidopsis cyclic phosphodiesterase	LPZ078	Novel
LPZ017	Novel	LPZ079	Novel
LPZ018	Novel	LPZ080	pM,dM:acyl-CoA-binding protein
LPZ019	pH:60S ribosomal protein L5	LPZ081	Novel
LPZ020	Novel	LPZ082	Novel
LPZ021	Novel	LPZ083	pL:putative pol polyprotein
LPZ022	dL:human EST sequence 003-T4-12	LPZ084	pM:acyl-CoA-binding protein
LPZ023	dH:EST sequence similar to insulin 1	LPZ085	pH:dynamin like GTP-binding protein
LPZ024	pL:lamprey neurofilament subunit NF-180	LPZ086	pM:acyl-CoA-binding protein
LPZ025	dH:5S rRNA gene	LPZ087	Novel
LPZ026	Novel	LPZ088	Novel
LPZ027	Novel	LPZ089	Novel
LPZ028	Novel	LPZ090	pL:acyl-CoA-binding protein
LPZ029	dL:oxygen evolving complex protein mRNA	LPZ091	dL:mRNA for LMW heat shock protein
LPZ030	Novel	LPZ092	Novel
LPZ031	Novel	LPZ093	Novel
LPZ032	dH:P. strobus mRNA for albumin 1	LPZ094	Novel
LPZ033	Novel	LPZ095	Novel
LPZ034	Novel	LPZ096	Novel
LPZ035	Novel	LPZ097	Novel
LPZ036	Novel	LPZ098	Novel
LPZ037	dM:oxygen evolvin complex protein mRNA	LPZ099	dL:rice cDNA tag
LPZ038	Novel	LPZ100	Novel
LPZ039	pL:maize actin, dL:actin gene	LPZ101	pL:poly-E rich protein, fertilin alpha protein
LPZ040	Novel	LPZ102	pL:C. elegans sequence ZK792.1
LPZ041	Novel	LPZ103	pL:C. elegans sequence C05C12.4
LPZ042	Novel	LPZ104	pL:pyruvate formate-lyase activating enzyme
LPZ043	Novel	LPZ105	Novel
LPZ044	Novel	LPZ106	Novel
LPZ045	pH,dH:rac like GTP binding protein	LPZ107	pL:yeast protein in SMY1-MUD2 intergenic region
LPZ046	Novel	LPZ108	Novel
LPZ047	Novel	LPZ109	Novel
LPZ048	pL:a yeast protein related to TFIIB	LPZ110	Novel
LPZ049	dH:starch synthase	LPZ111	Novel
LPZ050	dH:starch synthase	LPZ112	pH,dH:oxygen evolving complex protein
LPZ051	Novel	LPZ113	Novel
LPZ052	pH:dynamin like protein (cell plate formation)		



LPZ114	pL:myosin heavy chain	LPZ170	pL:follicular variant translocation protein 1
LPZ115	pM:many proteins including pherophorin and nuclear proteins, dM:mouse alpha-2 antiplasmin gene etc.	LPZ171	pL:adenylyl cyclase isoform DAC9
LPZ116	pH:translation EF-1-alpha	LPZ172	Novel
LPZ117	Novel	LPZ173	pL,dL:60S ribosomal protein L35
LPZ118	pL:lipase precursor, dH:rice cDNA tag C2611_1A	LPZ174	Novel
LPZ119	Novel	LPZ175	pL:nitrate reductase
LPZ120	pL:Bacteriodes thetaiotaomicron outer membrane protein	LPZ176	pH,dH:serine protein kinase
LPZ121	pM:C. elegans sequence ZK792.1, dH, rice cDNA tag E60576_1A	LPZ177	pL:Epstein-Barr nuclear antigen 2
LPZ122	Novel	LPZ178	Novel
LPZ123	pL:C. elegans sequence C05C12.4	LPZ179	Novel
LPZ124	pL:Bacteriodes thetaiotaomicron outer membrane protein	LPZ180	pH,dH:60S ribosomal protein L35
LPZ125	pL:African swine fever virus	LPZ181	Novel
LPZ126	pH:50S ribosomal protein L20	LPZ182	Novel
LPZ127	Novel	LPZ183	pH:60S ribosomal protein L35
LPZ128	Novel	LPZ184	pH:60S ribosomal protein L35
LPZ129	pL:C. elegans sequence C05C12.4	LPZ185	Novel
LPZ130	Novel	LPZ186	Novel
LPZ131	Novel	LPZ187	pH:60S ribosomal protein L35
LPZ132	pM,dM:acyl-CoA binding protein	LPZ188	pH:60S ribosomal protein L35
LPZ133	pL:yeast SLS1 protein	LPZ189	Novel
LPZ134	Novel	LPZ190	Novel
LPZ135	pM:C. elegans sequence ZK792.1, dH:rice cDNA tag E60576_1A	LPZ191	Novel
LPZ136	Novel	LPZ192	dH:Arabidopsis cDNA clone TAP0235
LPZ137	Novel	LPZ193	pH:60S ribosomal protein L35
LPZ138	Novel	LPZ194	Novel
LPZ139	Novel	LPZ195	pH:symbiosis related protein
LPZ140	Novel	LPZ196	pL:NADH ubiquinone oxidoreductase chain 2
LPZ141	Novel	LPZ197	pL:gibberellin related protein
LPZ142	Novel	LPZ198	pH:alpha tubulin
LPZ143	pM,dM:60S ribosomal protein L35	LPZ199	Novel
LPZ144	Novel	LPZ200	Novel
LPZ145	Novel	LPZ201	pL:phenylalanyl-tRNA synthetase beta chain
LPZ146	Novel	LPZ202	pL:late embryogenesis abundant protein
LPZ147	Novel	LPZ203	Novel
LPZ148	Novel	LPZ204	Novel
LPZ149	Novel	LPZ205	Novel
LPZ150	pL:gibberellin regulated protein GAS5	LPZ206	Novel
LPZ151	Novel	LPZ207	pL:plasma membrane ATPase
LPZ152	Novel	LPZ208	Novel
LPZ153	Novel	LPZ209	Novel
LPZ154	Novel	LPZ210	Novel
LPZ155	Novel	LPZ211	Novel
LPZ156	Novel	LPZ212	pL:histone H3 gene
LPZ157	Novel	LPZ213	Novel
LPZ158	Novel	LPZ214	Novel
LPZ159	Novel	LPZ215	Novel
LPZ160	Novel	LPZ216	pL:late embryogenesis abundant protein
LPZ161	Novel	LPZ217	Novel
LPZ162	pL:Arabidopsis ERECTA gene	LPZ218	pL:histone H3 gene
LPZ163	Novel	LPZ219	Novel
LPZ164	Novel	LPZ220	Novel
LPZ165	Novel	LPZ221	Novel
LPZ166	Novel	LPZ222	pL:chorion protein
LPZ167	Novel	LPZ223	pL:alpha amylase
LPZ168	Novel	LPZ224	Novel
LPZ169	Novel	LPZ225	pH:60S ribosomal protein L35
		LPZ226	Novel
		LPZ227	Novel
		LPZ228	pM:WD40 repeat type I transmembrane protein
		LPZ229	Novel
		LPZ230	Novel
		LPZ231	Novel



LPZ232	pH:NADP dependent oxidoreductase P2	LPZ277	Novel
LPZ233	pL:NADH ubiqinone oxidoreductase chain 5	LPZ278	Novel
LPZ234	Novel	LPZ279	Novel
LPZ235	Novel	LPZ280	pL:Arabidopsis hypothetical protein
LPZ236	pH:60S ribosomal protein L35	LPZ281	Novel
LPZ237	Novel	LPZ282	Novel
LPZ238	Novel	LPZ283	Novel
LPZ239	pH:oxoglutarate/malate translocator precursor	LPZ284	Novel
LPZ240	pH,dH:IAA-amino acid hydrolase	LPZ285	Novel
LPZ241	pL:neurofibromin	LPZ286	Novel
LPZ242	pH,dH:ubiquitin-like protein	LPZ287	pL:insect intestinal mucin IIM14
LPZ243	Novel	LPZ288	pH:cystathionine gamma-synthase
LPZ244	pH:small nuclear ribonucleoprotein Sm D3	LPZ289	dM:arabidopsis cDNA tag SASF004
LPZ245	pH,dH:ubiquitine-like protein	LPZ290	pH:60S ribosomal protein L35
LPZ246	pL:heme O synthase	LPZ291	pH:60S ribosomal protein L35
LPZ247	pH:protein kinase	LPZ292	pH:60S ribosomal protein L35
LPZ248	pH:malate oxidoreductase	LPZ293	pL:Arabidopsis hypothetical protein
LPZ249	pM:voltage-dependent anion channel protein	LPZ294	pL:Arabidopsis hypothetical protein
LPZ250	Novel	LPZ295	pH:Arabidopsis hypothetical protein
LPZ251	Novel	LPZ296	pH:Arabidopsis hypothetical protein
LPZ252	Novel	LPZ297	pH:60S ribosomal protein L5
LPZ253	pH:protein kinase	LPZ298	pH:60S ribosomal protein L5
LPZ254	pH:protein kinase	LPZ299	pL,dM:actin capping protein alpha subunit
LPZ255	Novel	LPZ300	pH,dH:vacuolar processing enzyme precursor
LPZ256	pL:latent transforming growth factor-beta binding protein	LPZ301	Novel
LPZ257	pL:Leishmania major glycoprotein 96-92	LPZ302	Novel
LPZ258	pL:HMG box containing protein 1	LPZ303	Novel
LPZ259	Novel	LPZ304	pL:Na ⁺ /H ⁺ antiporter (napA-2)
LPZ260	Novel	LPZ305	Novel
LPZ261	pL:ancient ubiquitous protein AUP46 precursor	LPZ306	pM:probable 40S ribosomal protein S30
LPZ262	pL:ancient ubiquitous protein AUP46 precursor	LPZ307	Novel
LPZ263	pM:Arabidopsis hypothetical protein	LPZ308	Novel
LPZ264	Novel	LPZ309	Novel
LPZ265	pL:transcriptional regulator IE63	LPZ310	pM:carrot B2 protein-like
LPZ266	Novel	LPZ311	pL:oleoyl acyl carrier protein thioesterase
LPZ267	pH:protein kinase	LPZ312	Novel
LPZ268	Novel	LPZ313	Novel
LPZ269	pH:60S ribosomal protein L35	LPZ314	pL:glutamic acid rich protein precursor
LPZ270	pH:heat shock protein 70	LPZ315	Novel
LPZ271	Novel	LPZ316	Novel
LPZ272	pL:Ecdysome-inducible protein E75	LPZ317	Novel
LPZ273	Novel	LPZ318	pM:ribosomal protein L23a
LPZ274	Novel	LPZ319	pM:ribosomal protein L23a
LPZ275	Novel	LPZ320	Novel
LPZ276	pL:yeast probable membrane protein	LPZ321	pL:RNA polymerase I, II, III 17.1 KD subunit



High density array:

To detect the expression of all of our cloned cDNAs in the embryos, the cDNAs need to be blotted on a piece of membrane. Because we have a large number of cDNAs, regular dot or slot blotting would produce a membrane too large to handle. The cDNAs have to be in a high density array. In a high density array, the individual cDNA dot is very small, and is sometimes difficult to distinguish from signal noise in the hybridization. To overcome this problem, we blotted each cDNA four times on the membrane. The 326 unique cDNAs were denatured and blotted on a 11mm x 6.5mm nylon membrane as 1304 dots. The membrane was then hybridized to probes made from mRNA extracted from embryos. An illustration of this procedure is shown in Figure 1. The small amount of cDNA is present in all dots, the difference in signal intensity reflects the different amounts of specific mRNA in different embryo stages.

Currently, the expression patterns of the genes represented by these cDNAs have been examined in stages 1-9 of somatic embryos and stages 1-9.10 of zygotic embryos. Differences and similarity in gene expression has been detected (Figure 2). In Figure 2 we see that most quartets vary little in signal intensity in the three zygotic embryo stages and one somatic embryo stage shown. However, certain quartets show a very strong signal intensity with one particular embryo RNA, indicating elevated levels of RNA in embryos at that stage of development.

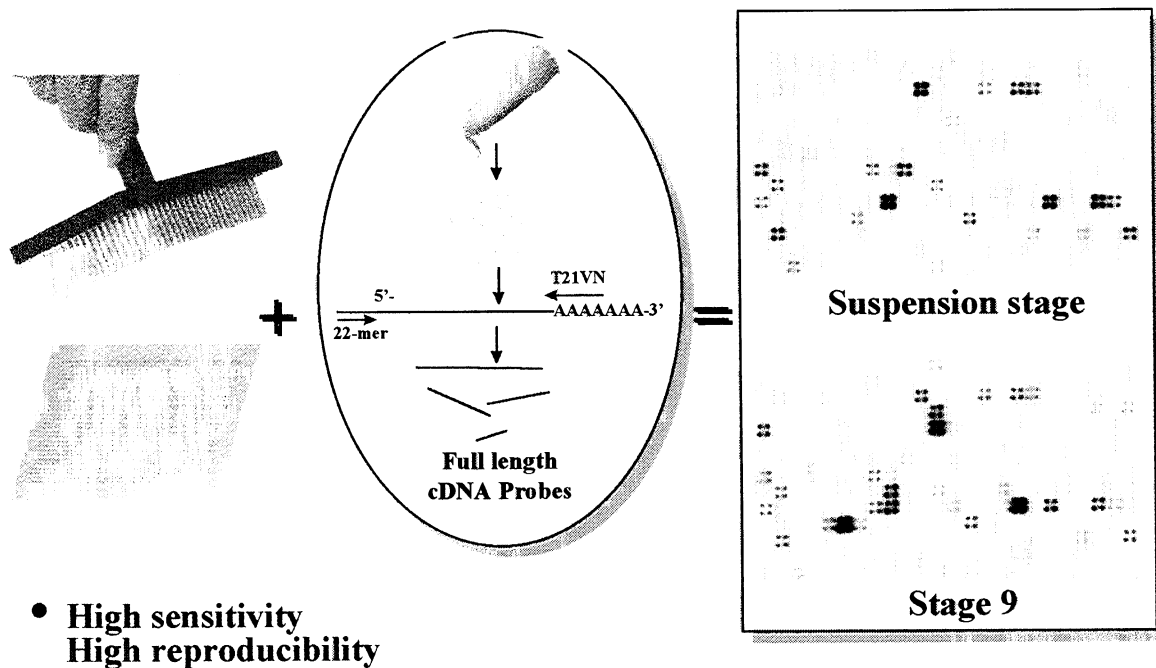


Figure 1. High-density array Southern with amplified full length cDNA



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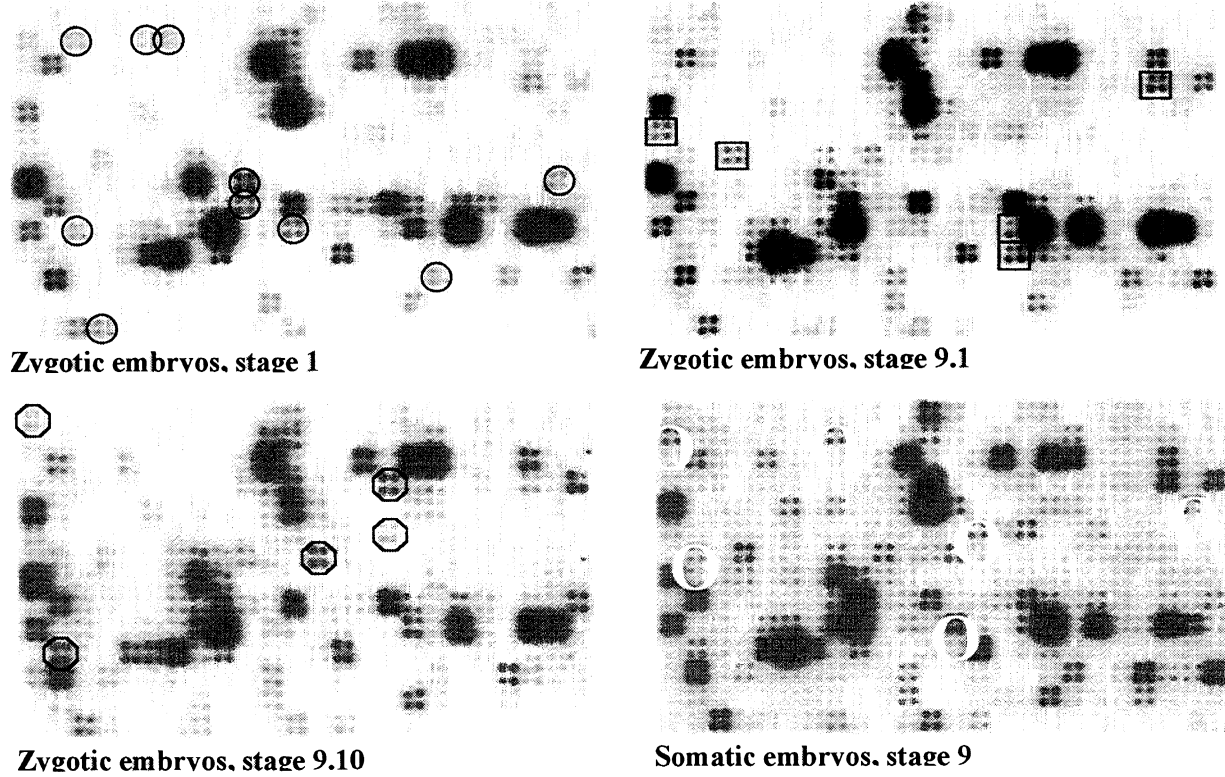


Figure 2. Comparison of gene expression patterns in zygotic embryos different stages and in somatic embryos. Circles = early genes; square = mid stage genes; octagon = late genes; letter O=somatic embryo genes



Isolation and characterization of "Pinine", a vicilline like seed storage protein from Loblolly pine (*Pinus taeda*).

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The expression of seed storage protein genes is specifically induced during seed development and restricted to the embryos and/or the endosperm[1]. It occurs primarily in specialized storage cells in the embryonic axis, cotyledons, and the endosperm of developing seeds but never in mature vegetative organs [2]. For an example, synthesis of the 2S protein, which is encoded by a multi-gene family was shown to be restricted to the latest stages of seed development and was correlated with the accumulation of the corresponding mRNA [8]. Also, regulatory domains required to establish the specific expression pattern of these storage protein genes were identified in their promoter by deletion and gain of function experiments in transgenic plants [3,4,5,6,7].

Thus, these genes represent an interesting model for studying the mechanisms of tissue and development specific expression.

In angiosperms and Gymnosperms the maximum accumulation of seed storage proteins and abundant synthesis of oil, and starch were found in the second phase of embryogenesis (mid embryogenesis), initially consists of rapid cell divisions to define the cotyledons. The gene families encoding the main storage proteins have been characterized in numerous plant species [9,10,11]. Storage proteins are classified into four groups according to their solubility properties. Albumins are water soluble; Globulins are salt soluble; Glutelins are soluble in acids, alkali ionic detergents, and urea-containing solutions; and the Prolamin are alcohol soluble. The globulins are usually the most prevalent class in legumes and oats, and the glutelins and prolamins are the major forms of cereal storage proteins [12,13]. However, the accumulation of proteins and their quantitative and qualitative properties have not been studied in details in majority of Gymnosperms and non vascular plants.

In a recent study, a contrasting storage protein synthesis and messenger RNA accumulation were nicely illustrated during development of zygotic and somatic embryos of Alfalfa (*Medicago sativa L.*)[13]. Superficially, somatic embryos mimic the developmental stages of zygotic seeds (globular, heart, torpedo, cotyledonary stages); however, despite these gross morphological similarities somatic embryos sometimes exhibit other features suggesting an aberrant development (truncated cotyledonary development, precocious germination, recalling, multiple or fused cotyledons, inability to germinate, etc.).

Comparison between somatic and zygotic embryos have shown that the later accumulate some seed storage products and can be induced to become desiccation tolerant under appropriate culture conditions. However, few studies have been done on the physiological and biochemical changes occurring somatic embryogenesis in parallel with studies of zygotic embryogenesis. In particular, seed-specific storage proteins, because of their nature and abundance, are expected to be useful markers for studying gene expression in these contrasting embryogenic systems.

The consensus of opinion has been that storage protein accumulation in seed is temporally and spatially regulated, primarily at the level of gene transcription. This conclusion is based largely on the absence of seed storage protein mRNA from nonseed tissues, and, also the observed coincidence between the period of maximum storage protein synthesis in developing seeds and mRNA accumulation as determined by northern blot techniques. More direct analyses of transcriptional rates using *in vitro* nuclear run-off experiments have confirmed that transcriptional activity is primarily determinant of changing mRNA and protein populations in seeds. Nonetheless, it has become increasingly clear that steady state mRNA levels are influenced significantly by posttranscriptional processes (mRNA processing, transport, stability). Also, translational efficiency and/or selective protein degradation may be important in determining the extent of storage protein accumulation in seeds. The relative importance of these factors in effecting storage protein accumulation in somatic and zygotic embryos may vary with the specific storage protein, the stage development, the tissue (axis or cotyledon), and the nutritional conditions. The magnitude of each of these influences in determining the observed storage protein complement of somatic (artificial) embryos remain to be determined.

The present report is mainly about the isolation and characterization of one of the vicilline like seed storage proteins "Pinine" from Loblolly pine embryos.

Experimental plan and results:

DNA library construction and screening

We have already cloned and sequenced almost all the full length clone of Loblolly Pine "Pinine" Vicilline like seed storage protein gene. We have prepared the cDNA library from Zygotic embryos, using cap finder PCR cDNA library construction kit from Clontech laboratories. The cDNA library was consisted with 2.5×10^6 independent clones in Lambda gt11 vector. 60000 Lambda clones from the library were screened with a 1.4 Pinine fragment previously cloned by PCR. Several putative cDNA clones were isolated and labeling and hybridization and washing were performed according to Church and Gilbert protocol.

We have also constructed a "Genome walker library" (Clontech CA) in order to isolate upstream promoter fragments of Pinine gene. Several putative promoter fragments has

been cloned and sequenced. Importance of analyzing promoter fragments will be discussed in detail.

Southern Hybridization

CsCl purified Loblolly Pine DNA and several other plant DNA, such as Arabidopsis, Tomato, and Cotton wood was used to perform southern hybridization. Genomic DNA was digested with restriction endonuclease EcoR1 and Bam H1 and hybridized with a radioactive probe of 1.4 Kb Pinine gene. Eight bands of different sizes (more than 1.6 Kb) was discovered in the autoradiograph. However, only one band was visible in Tomato. This clearly indicate the presence of multi copy number of a single gene or multicopy numbers of different alleles of the same gene. This is a clear indication for the fact that gymnosperms contain multi copies of any given gene compare to angiosperms.

Pinine gene expression in Somatic and Zygotic embryos

Pinine gene expression in somatic (stage 1 to stage 9) and zygotic (stage 1 to stage 9 and stage 9.1 to stage 9.12) was studied. Data from these experiments will be discussed in detail.

Behavior of Pinine gene in different stress conditions

We were also interested in investigating the general stress response (somatic, heat, aerobic, anaerobic and nutritional) on the accumulation of Pinine like seed storage protein in cell suspension as well as different stages of somatic and zygotic embryos. In order to do so, we have decided to carry out the following experiments. Loblolly Pine cell suspension culture #260 was grown in 37C' in order to provide the heat stress. Several aliquots were taken after 3 hours, 6 hours, 9 hours, 15 hours, 15 hours plus one hour drying and then prepared RNA immediately to study the gene expression .

As the control we used RNA from the cell suspension grown in room temperature. Northern hybridization data showed that there is no gene expression in the control (cell suspension grown in room temperature), as well as RNA isolated from the samples those were grown 3 hours 6 hours and 9 hours in 37C'. However, those cells grown in a longer period, 15 hours in 37C' showed the accumulation of RNA. These data support with the previously published data which indicates " osmotic stress could induce storage protein and storage protein transcript accumulation" in somatic and zygotic embryos. However, no one has yet studied a similar relationship between seed storage protein accumulation and stress in cell suspension culture. We will present several possible arguments for this issue.

Future experiments

Currently we are performing the following experiments.

- a) Isolating different Pinine promoter fragments to seek whether they carry sequence elements, homologous to any heat-shock elements or other stress elements.

- b) Ribonuclease protecting assay experiments with different stages of somatic and zygotic embryos.
- c) Insitu hybridization to investigate the Pinine protein accumulation in specific stages in somatic and zygotic embryos.

Partial copy of a nucleotide alignment of "Pinine" gene with P. glauca vicilin-like protein.

emb|X63191|PGVICSP P.glauca mRNA for vicilin-like stora... 2040 1.9e-159 1
 gb|L47744|PIAEMB18R Picea glauca vicilin-like protein (E... 1995 1.1e-155 1
 emb|Z50791|ZFVICLN7S Z.furfuracea mRNA for vicilin (7S gl... 533 5.3e-40 2
 emb|Z54365|ZFVICGN Z.furfuracea gene for vicilin 185 3.8e-05 1
 emb|X63191|PGVICSP P.glauca mRNA for vicilin-like storage protein, Length = 1590

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Culture Cycling and Tissue Culture Decline: pH Cycling in Loblolly Pine Suspension Cultures and Associated Gene Expression

Michael Sullivan, Byron Waldrop, Jerry Pullman and John Cairney

Cycling in liquid culture somatic embryos is defined by fluctuations in culture growth as indicated by settled cell volume, embryo stage and appearance, and cotyledonary yield upon plating. An example of a culture in a cycling mode appears in Figure 1. In this plot of settled cell volume versus time, the culture volume at a peak can be more than 150% of the volume at the preceding valley. In these experiments carried out at IPST, a regular three week interval was observed between the peaks. Cycling is often the part of a larger trend of culture decline, with successive yields diminishing until little or no growth of a suspension culture is observed and that culture is lost.

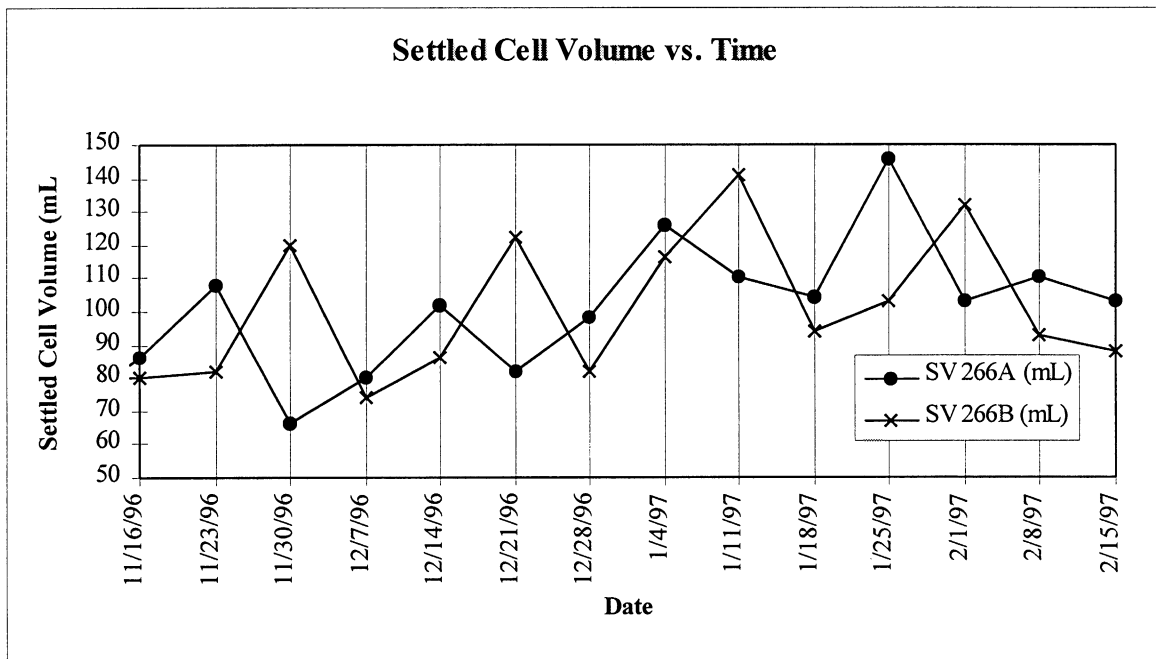


Figure 1. Settled Cell Volume vs. Time for early stage somatic embryos of *Pinus taeda* Genotype #266 culture A and B. Fluctuations in settled cell volume appear at regular three week intervals.

Currently there is not an adequate explanation for the cycling phenomena. It is unlikely that environmental factors are causing cultures to cycle. Other cultures that are maintained under identical conditions do not show the same magnitude or regularity of fluctuations. It has been reported that genotypes and cultures with poor quality embryos

and low maturation rates cycle through growth fluctuations, but a relationship between cycling and culture decline is yet to be established. The fluctuations in measured growth parameters must be related to growth mechanisms within the cell that are possibly triggered by changes in gene expression.

There is a need to characterize the types of changes that are a result of the cycling phenomenon. Changes in growth patterns that are related to the cycling phenomenon may make it difficult to identify changes that come as a result of alterations to protocol. Also, variations in gene expression within a culture will be of great interest to investigators who search for differences in gene expression between “high” and “low” quality embryos.

A project was initiated in 1996 to examine cycling in loblolly pine suspension cultures with a view to characterizing the phenomenon further with respect to cell volumes, medium osmolarity and pH and to identifying genes whose expression correlated with peaks or troughs in the cycle. Such genes may act as markers for phases in a culture cycle, may indicate the health of a culture and, if functions can be ascribed to their encoded proteins, inferences about the embryo's metabolic state may be made.

Initial reports about the variation in cell volume over time have been reported previously (PAC Report March 1997 and Fig.1). This project revealed a cycle of pH fluctuation which preceded volume changes by one week. This observation was based on a limited number of measurements however pH cycling has now been investigated further and our most recent findings are reported below.

Tissue Culture Techniques

Two different *Pinus taeda* genotypes, numbers 260 and 266, were used to assess both the physical and biochemical implications of tissue culture decline. Both genotypes have initiated somatic embryos and have been maintained in liquid media number 16 for 121 days prior to my receiving them. The two genotypes were subdivided during the first week following the initiation of somatic embryogenesis, so that duplicates of each are being maintained. The subdivided genotypes are labeled 260A, 260B, 266A and 266B respectively.

Once per week the physical characteristics; settled volume, pH, osmolarity, and developmental stage, of each culture will be assessed. During this same time a portion of cells will be transferred to solid developmental media number 240 to determine their ability to produce cotyledonary embryos. Others will be transferred to fresh liquid media number 16 and the remaining cells frozen with liquid nitrogen and stored for mRNA analysis at a later date. The procedure for cell transfer and analysis of physical characteristics is outlined below.

- Entire liquid culture (300 mL) is transferred to a graduated cylinder and cells are allowed to settle for 20 minutes. At the end of 20 minutes the volume occupied by the cells is recorded as the settled cell volume.
- A 5 mL aliquot of cells is removed from the settled volume and 1 mL placed on each of 5 prepared sterile petri dishes containing 10 mL of developmental media number 240. Each culture will be maintained on media number 240 for 9 weeks, being transferred to fresh media at the end of 3 and 6 weeks.
- A 30 mL aliquot of cells is removed from the settled volume and placed in 270 mL of fresh liquid media number 16.
- A 5 mL aliquot of cells is removed from the settled volume and placed on a petri dish for developmental stage determination. Stages are determined using the IPST staging system (Pullman, 1994).
- The remaining cells are separated from the liquid media by suction filtration and frozen using liquid nitrogen. Each sample is then stored at -70 °C until need for analysis of mRNA.
- The liquid media isolated following filtration is analyzed for vapor pressure osmolarity and pH.

Observed Culture pH Cycling

Early in the spring quarter of 1997 it was observed that the pH of liquid suspension cultures underwent a regular cycling pattern. The cycling occurred such that a peak in pH preceded the decline in settled cell volume by one week. Low settled cell volume was typically accompanied by a trough in the pH cycling. This behavior was observed in each of the four *Pinus taeda* cultures being maintained (two cultures of genotype 260 and two of 266). Genotype 266A displayed the most regular cycling pattern as well as the greatest exaggeration between pH peaks and troughs, for this reason it was targeted for investigation. The pH cycling for genotype 266A can be seen in figure 2, arrows indicate the weeks of particular interest.

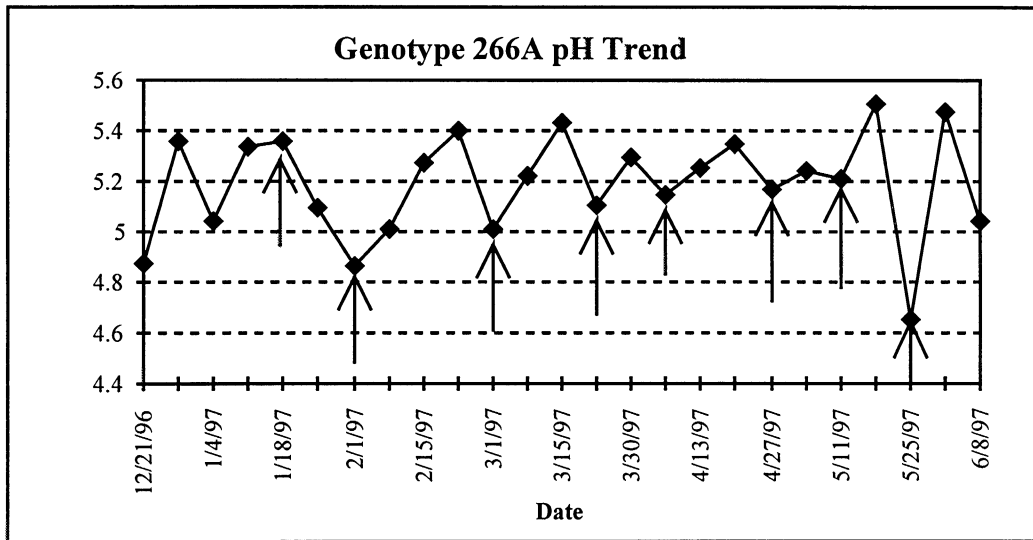


Figure 2. pH trend for genotype 266A, arrows indicate troughs in settled cell volume.

Molecular Analysis

As stated earlier, by determining gene expression at different points in the cultures' pH cycle, and by identifying specific genes whose expression is pronounced at that time, we hope to gain insight into physiological processes which are occurring. We have employed differential display to view gene expression at different points in the pH cycle and have now cloned a number of cDNAs which correspond to mRNAs present in increased amounts in either pH peaks or troughs.

Differential Display

Somatic embryos were harvested, frozen using liquid nitrogen and stored at -70°C . A portion of the frozen tissue was removed from the freezer and allowed to thaw on ice. mRNA was isolated using an adapted magnetic separation method as developed and adapted in our laboratory. The adapted method for somatic embryos requires a minimal amount of tissue and has proven to be extremely sensitive and rapid. The procedure involves lysing the cell wall, isolating mRNA using oligo(dT) beads and performing reverse transcription to generate mRNA templates. The amount of oligo(dT) beads used for the isolation of mRNA has been reduced from $50\ \mu\text{l}/\text{sample}$ to $8\ \mu\text{l}/\text{sample}$ to accommodate the small amount of mRNA in the sample. Excess oligo(dT) may inhibit subsequent PCR reactions resulting poor resolution and ladder like appearance of bands. Magnetic oligo(dT) beads, lysis, binding and washing buffers were all provided by Dynal (mRNA DIRECT kit, Prod. no. 610.11). Products used for reverse transcription, dNTP's and arbitrary primer, were provided by GenHunter Corporation (RNAmappingTM, 1994).

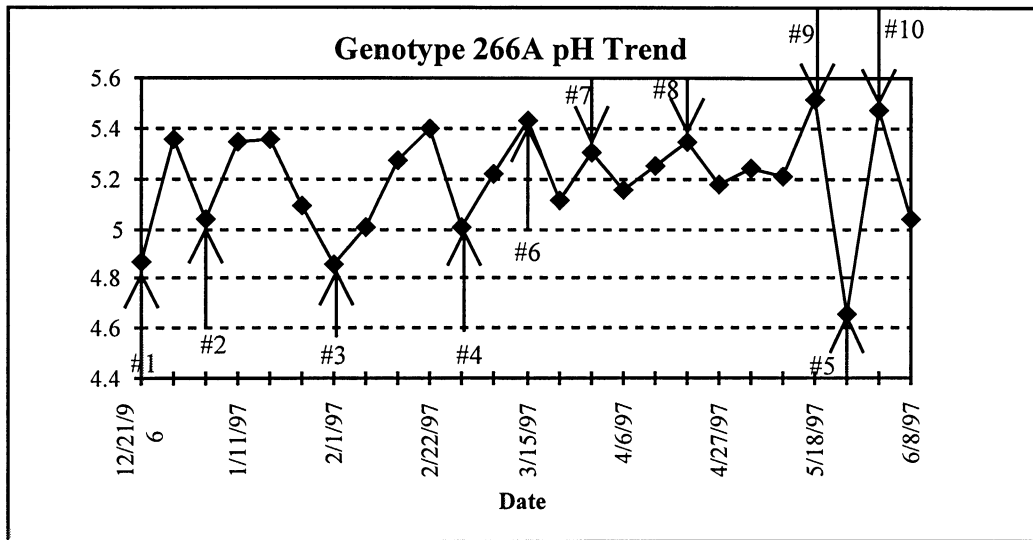


Figure 3. Genotype 266 pH peaks and troughs selected for differential display. mRNA was isolated from weeks indicated by arrows, number corresponds to the lane assignment in differential display gel #105.

PCR was carried out using the RNAmapping™ protocol designed by GenHunter Corporation (RNAmapping™, 1994). The primers T₁₂MT, T₁₂MG, T₁₂MC, T₁₂MT, AP3 (5'-AGGTGACCGT-3') and AP-1 (5'-GGTACTCCAC-3') were used in five different combinations along with RT products isolated from pH peaks and troughs. PCR was performed as 94° C, 30 sec --- 40° C, 2 min --- 72° C, 30 sec for 40 cycles --- 72° C, 5 min --- 4° C. PCR products (5µL) were combined with 1µL loading dye (GeneHunter) and heated at 80° C for two minutes. 1.7 µL of the mixture was loaded onto a 6% polyacrylamide denaturing gel. Samples were electrophoresised for 3-4 hours at a constant voltage and temperature of 2000 V and 42° C respectively.

To increase confidence in the reproducibility of the differential display, two different quantities of cDNA were used for the reaction. Where similar patterns were seen in each lane a band might be chosen for further analysis. 'Peaks' were run alongside each other as were 'Troughs'. We looked for bands present in all 'Peak' lanes but absent from 'Trough' lanes and vice versa. Some band could be identified which fulfilled or nearly fulfilled these requirements (Fig. 4). In some cases a band might be absent in 'peaks' and present in most 'troughs', the 'trough' in which it was absent was often a 'high trough' such as trough 2 or 4 (Fig.3). Such bands were excised for further study and confirmation. Thus some of our bands may be responsive to an absolute pH threshold rather than a point in the cycle. We believe that these will contribute to our understanding of gene expression during cycling.

Genotype 266A, Primers: T12MT + AP-3

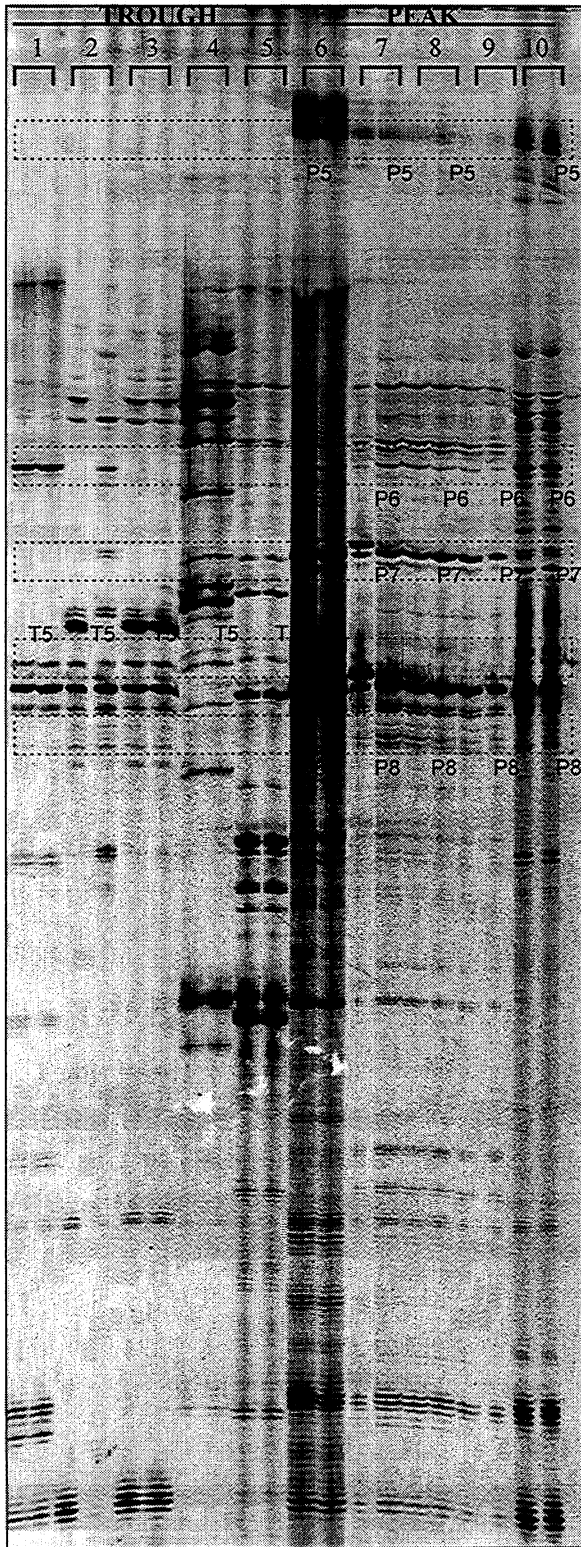


Figure 4. Differential Display Gel Showing bands which were cut out as potentially correlating with pH Peaks or Troughs.

Excising, Amplifying and Purifying Specific cDNA

Bands of interested were excised from the gel by aligning the autoradiograph with the gel and cutting the bands using a scalpel and 1 μ L of sterile water. PCR was used to amplify the excised bands. PCR products were run a 2% agarose gel for 15 minutes at a constant voltage of 80 V, to confirm that the band had been amplified. The remainder of the product was purified using a chroma spin-100 column (Clontech, 1996).

PCR cocktail for band amplification:

- 23.65 μ L dH₂O
- 4.00 μ L T₁₂MN (10 μ M)
- 4.00 μ L Arbitrary primer (2 μ M)
- 3.20 μ L dNTP's (250 μ M)
- 4.00 μ L 10x PCR buffer (Perkin elmer)
- 0.40 μ L Taq polymerase (Perkin elmer)

Cloning selected cDNA and colony PCR

Column purified cDNA was ligated overnight into the pCR 2.1 vector (Invitrogen, 1996). Ligation products were transformed into competent cells provided by Nanfei Xu. Transformed competent cells were plated unto LB media containing X-gal and IPTG and incubated at 37° C for 16 hours. White colonies were selected and colony PCR performed to confirm cDNA insertion.

PCR cocktail for colony PCR:

- 10.2 μ L dH₂O
- 2.0 μ L Lgh primer
- 2.0 μ L Rgh primer
- 1.6 μ L dNTP's (250 μ M)
- 2.0 μ L 10x PCR buffer (Perkin elmer)
- 0.20 μ L Taq polymerase (Perkin elmer)



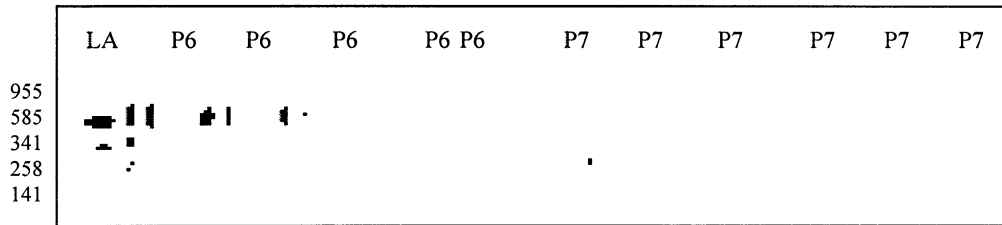


Figure 5. Colony PCR of putative clones of bands excised from differential display gels

Figure 5 shows PCR results for several clones of two Peak-specific bands. Since excised bands may be heterogeneous the number of different sequences represented in these clones needs to be determined. Aliquots of colony of PCR were analyzed by restriction enzymes to further classify the clones. The digestion was carried out as indicated below using two restriction enzymes; Hha I and Hind III.

Rxn 1

16.9 uL dH₂O
 0.10 uL Hha I (20 U/uL)
 3.00 uL NEB buffer #2
 10.0 uL colony PCR product

30.0 uL / Rxn

Incubated @ 37 C for 1 hour and then added second restriction enzyme

Rxn 2

0.10 uL Hind III (20 U/uL)
 3.00 uL NEB buffer #2

3.10 uL / Rxn

Continued incubation @ 37 for 2 hours



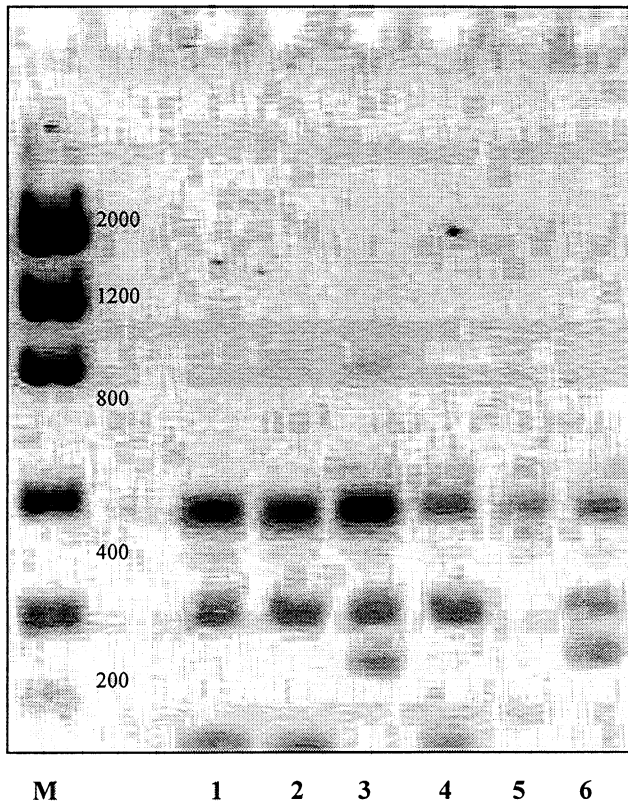


Figure 6. Digest of PCR products from clones of Band T5. Note three classes of clone and revealed by restriction digest; Class 1 - lanes 1, 2, 4; Class 2 - lanes 3,6; Class 3 lane 5. M is the DNA size marker lane.

The clones have been sorted into classes according to our results and their pattern of expression is being confirmed using Dot Array Southern (DAS, see Xu et al this report). Once confirmation of expression is obtained we will conduct Northern analysis over the range of the cycle using RNA from different suspensions. In this project, dealing with suspension cultures, there is sufficient material for each experimental point to allow Northern analysis to be conducted. This will give us sensitive assays to gene expression and allow us to determine the size of the mRNA. Once gene expression differences have been confirmed clones will be sequenced and similarities with known genes will be sought by GenBank searches.



cDNA Cloning of mRNAs Present Early in Zygotic Embryo Development

Vincent Ciavatta, Gerald Pullman and John Cairney

Identification of genes whose expression occurs early in embryogenesis and whose expression is confined to that period of embryogenesis will provide clues as to the crucial metabolic events which are occurring within the embryo and provide molecular markers to follow development in somatic and zygotic embryos. In this project we have focused on genes whose expression was confined to the earliest stages of embryogenesis and further was, if possible, restricted to a single stage (contrast this with the report of Xu et al which sought examples from early and late stages or embryogenesis including genes whose expression spanned several stages).

In work reported previously (Forest Biology PAC Report October 1997), mRNAs expressed at early stages of development were identified by differential display. A number of bands which appeared in samples from stage 1-3, but were absent in later stages were excised from the gel. The following report describes progress in cloning and identifying these bands.

I. Cloning and Classifying Bands 1 - 14 from Differential Display Gels

Bands were excised from gels, re-amplified and ligated to PCR cloning vectors as described earlier (Forest Biology PAC Report October 1997). All sequences were successfully reamplified. Restriction digest fingerprinting revealed several classes of clone arising from each band excised from the gel (Table 1). that were subsequently prepared for a Dot Array Southern (DAS).

II. DAS to Confirm Differential Expression

To confirm early expression of each distinct clone isolated above, we performed Dot Array Southern. The principle of the technique was explained in detail in Forest Biology PAC Report, October 1997 and is give in more detail in the report of Xu et al in this volume. The protocol details are given in the Material and Methods section at the end of this report. Denatured cDNAs were manually spotted onto two nylon membranes. One membrane was probed with labeled PCR cDNA derived from mRNA from zygotic stages 1 and 2 and the other membrane was probed with labeled PCR cDNA from zygotic stages 8 and 9.1. Results of the DAS (Figure 1) show 10 clones that were selected as deriving from genes whose expression is maximal in early stage embryos. In this assay, the intensity of the signal reflects the abundance of a particular mRNA species thus the darker the spot, the more abundant is that particular mRNA in the



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embryo. It should be noted, however, that the earlier stage probe was more radioactive than the late stage stage probe. Therefore, some portion of the more intense signals produced from the early stage blots has to be attributed to a hotter probe. Nevertheless, the ten clones selected on this basis were prepared for sequencing and further confirmation.

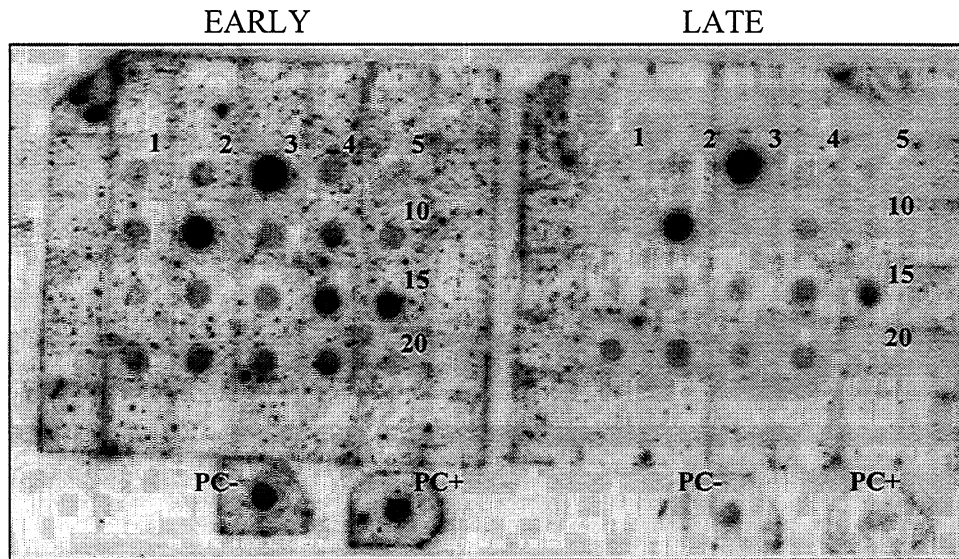


Figure 1. Autoradiogram of the DAS to test differential expression of clones listed in Table 1. Two identical blots were made. The blot on the left was hybridized to an early stage probe and the blot on the right was hybridized to a late stage probe. Pattern of dot blotting is indicated by the numbers. Dot 20 was a control cDNA from N. Xu that was not synthesized from the same AP and T₁₂ primers used for spots 1 - 19, hence hybridization was not expected. PC- & PC+ are described in Materials and Methods. The (-) was not exposed to UV, (+) was exposed to UV. Clones 1, 2, 4, 6, 8, 9, 10, 11, 18 and 19 were interpreted as being preferentially expressed in early stage zygotic embryos.

III. Sequencing and Homology

For sequencing, plasmid DNA of each clone was sent to the University of Missouri DNA Core Facility. It was reported that, in general, more plasmid DNA should be sent in the future as the signals from these bands were weak. Still, only one of the ten sequences was unreadable. Sequences das18 and das19 were pieces of the same cDNA with 18 being the longer fragment. Similarly, sequences das1 and das2 were pieces of the same cDNA with 2 being the longest.

Homology searches were done to compare these seven unique sequences with nucleic acid and amino acid sequences in the National Center for Biotechnology Information (NCBI) non-redundant (nr) BLAST data bases. Most cDNAs show very weak homology to known sequences at the nucleic acid and amino acid level. Two cDNAs do show some stronger homologies. The sequence with the most compelling homology, das6, shows a region of 61% amino acid identity (84% positives) over a



stretch of 98 amino acids (294 nucleotides) with a *Drosophilla* transmembrane protein (*des1*) that is required for spermatogenesis (Endo, et al., 1996). Similar levels of homology were found between *das6* and the mouse homologue of *des1* (Endo, et al., 1997) and an MLD gene that is a member of a fatty acid desaturase family (Cadena, et al., 1997). The sequence showing the next lowest P value was *das8*. This 193 nucleotide long cDNA has 77% nucleotide identity over a stretch of 43 nucleotides to an *N. tabacum* mRNA for a MADS-box protein. All other sequences have rather insignificant homologies as indicated by very large P values.

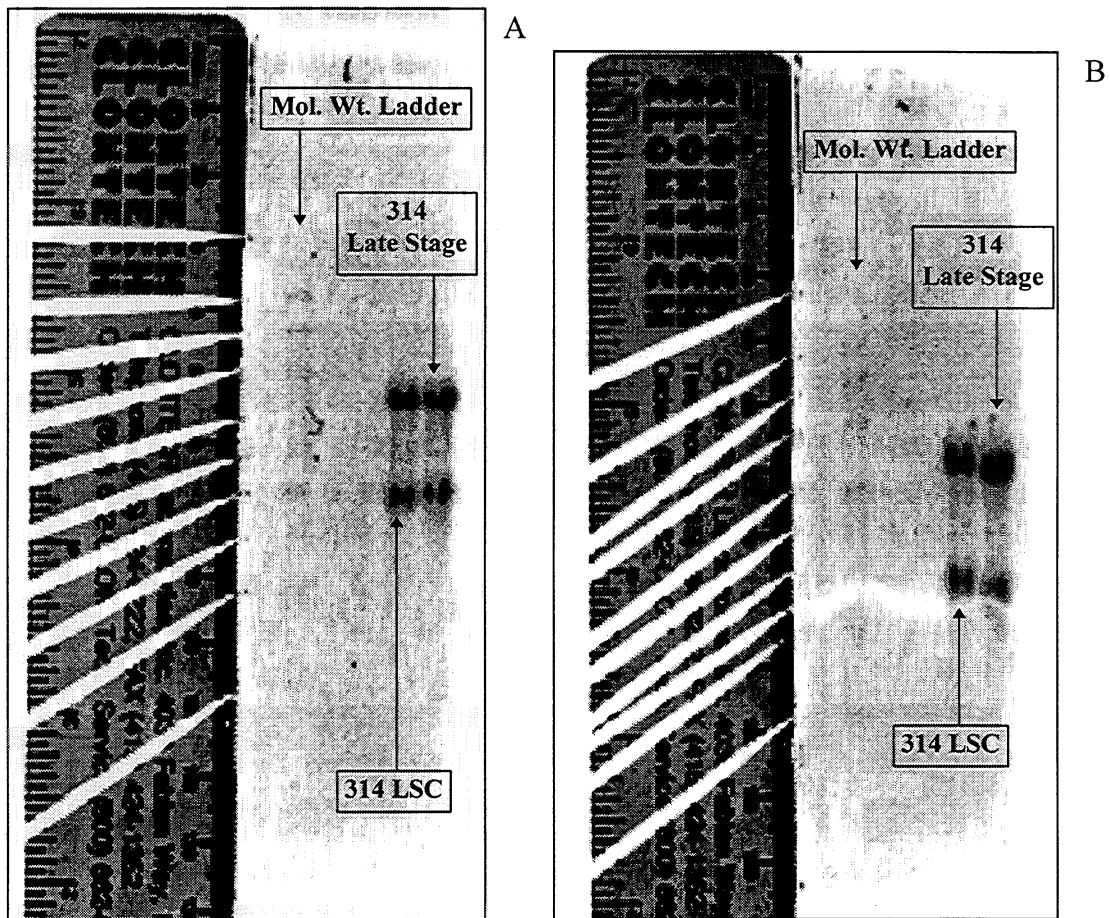
IV. Northern Blotting

Positive northern blots will provide two key pieces of data in characterizing putative differentially expressed sequences. One, evidence for relative abundance of mRNA in somatic LSC and late stage embryos (i.e., differential expression) can be interpreted from intensities of autoradiograms; confirmation of earlier results indicating differential expression. Northern Blots are possible where relatively large amounts of RNA can be isolated (5-10 microgram per sample). This is not possible for every stage of pine embryo since the quantity of embryos available is limited. In these experiments we therefore confined our study to RNA from liquid suspension culture and Stage 9 embryos, samples which were abundant and from which much RNA could be prepared.

In preparation for northern hybridizations, eight duplicate northern blots of genotype 314 LSC and late stage total RNA were made. Unique, cloned sequences from DDs 4609-35 & 43 as well as DD 4397-34 are candidates to be checked with these northern blots. To prepare cDNAs for probe synthesis, PCR was done on glycerol stocks of each clone, and the resulting DNA bands were purified and gel quantitated by comparing to a low mass ladder.

RNA was separated on a gel and stained with Ethidium Bromide to confirm equal loading (Fig.2 A and B). The two bands seen on the gel are ribosomal RNA molecules. To date, northern blots have been probed only with *das6*. At the time of writing, this blot is being exposed to film. See appendix for results of *das6* probe of northern blot.





Figures 2A & B. Photographs of the leftmost portions of gels used for northern blotting. See Materials and Methods for electrophoresis and transfer procedures. Gel slices were soaked two times for 20 minutes in 0.5M ammonium acetate and then stained for 40 minutes in 0.5M ammonium acetate with 0.5 ug/mL ethidium bromide. Each gel slice pictured has 4 uL of ladder, 7 ug of LSC RNA and 7 ug or Late Stage RNA. Gel B ran slower than C probably due to differences in wiring of gel tanks or buffer concentrations. Toothpicks were aligned with ladder markers to better visualize on the photograph.

das6 Probe of Northern Blot

Two blots were performed using *das6* as a probe as seen in Fig.3. In each case a stronger signal was observed in Liquid Suspension Culture compared to Stage 9 embryos. This indicates that while expressed in both stages of embryo development examined here, *das6* mRNA is more abundant in early stage embryos. This is what we expect for a cDNA which was selected originally as being expressed preferentially in early stage embryos (though in this case expression seems not to be exclusive to early stage embryos, at least in the somatic embryos examined here).

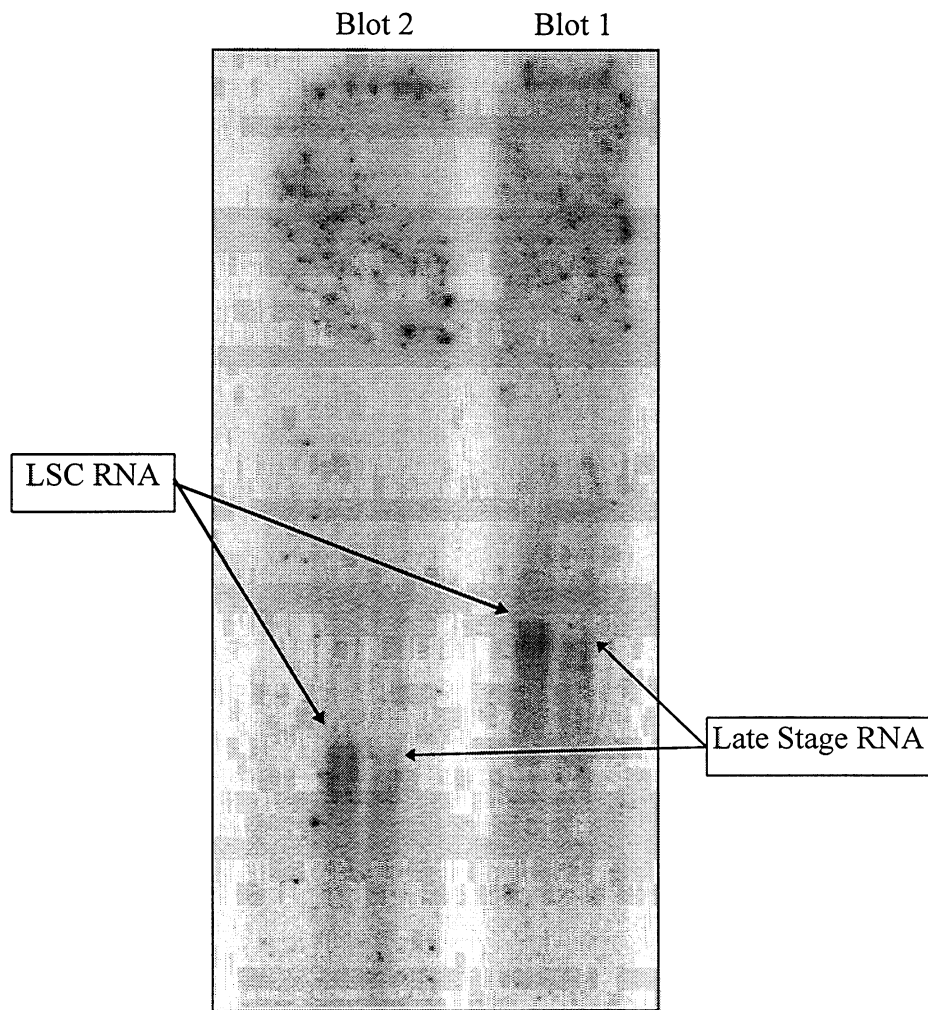


Figure 3. Photograph of *das6* probe of genotype 314 LSC and late stage RNA. See materials and methods for description of electrophoresis, transfer, and hybridization conditions. Two separate gels and transfers were performed. Two membranes were probed and exposed, one from each northern blot. Note the more intense signal seen from the LSC lanes. Membranes were exposed to film for approximately 41 hours.

V. RT-PCR for *das6*

As stated in the previous section, Northern Analysis is only possible where large amounts of RNA can be isolated. However we wish to follow expression of genes over the course of embryogenesis. Reverse Transcription-PCR (RT-PCR) requires little RNA as a starting substrate so is a useful technique for our studies. Data from RT-PCR will provide evidence that a particular mRNA sequence is present at a given stage of embryo development. In addition to being qualitative, there is also the potential to make the assay quantitative to show the abundance of a transcript. In the present case, steps have not been taken to construct a quantitative RT-PCR assay, but this may be addressed in the future. Primers specific to *das6* were synthesized (Table 2). For a first attempt at RT-PCR with these primers, first strand cDNA from earlier experiments was used. PCR resulted in the synthesis of a band that comigrates with the positive control band for UC5-



1036 zygotic embryos stages 1 - 9.1 and faintly in genotype 255 somatic embryo LSC and possibly late stage (Figure 4). There appears to be much greater expression in zygotic embryos and expression is throughout development. With somatic embryos expression is much greater in Liquid Suspension Culture than at later stages. These experiments are being repeated.

Table 2. Primer sequences for das6.

Primer Name	Primer Sequence
LPdes F (19 mer)	5'-AATGCATCCTATGGCTGGG-3'
LPdes R (20 mer)	5'-TCTCACGTCCACTTGAATGG-3'

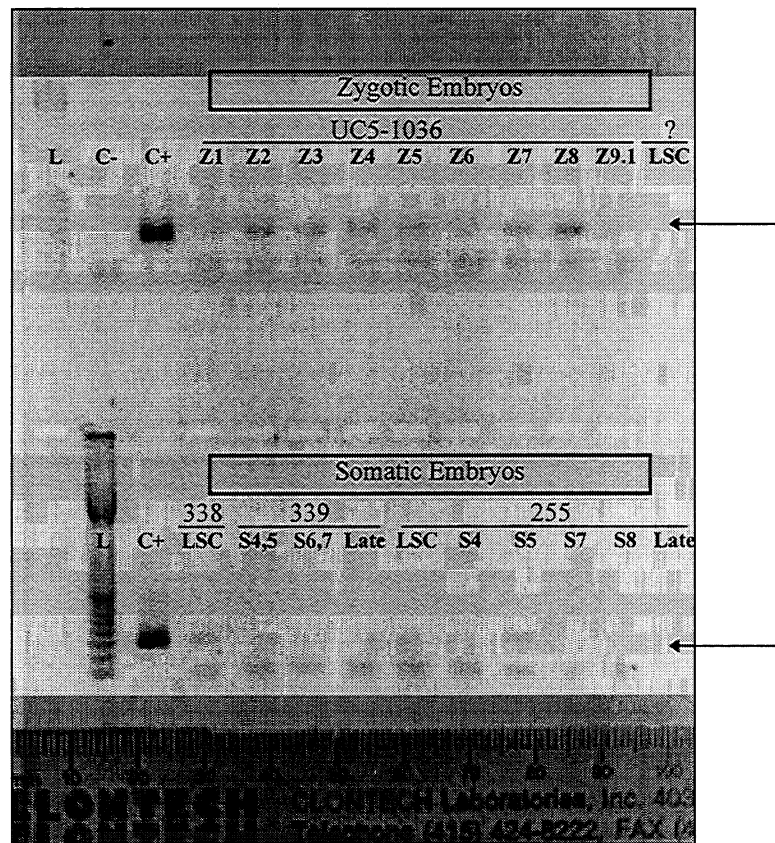


Figure 4. Photograph of RT-PCR results. See materials and methods for reagent concentrations and cycling conditions. Fifteen microliters of each PCR product was loaded per lane. Gel was run 60 Volts for 1 hour in 1X TAE buffer containing 0.5 ug/mL ethidium bromide. Ladder (L) is 100 bp ladder, 2 uL in the top half and 4 uL in the bottom half. Negative control and positive control are marked C- and C+, respectively. Tissue origin: UC5-1036 (zygotic), 338, 339, or 255 (somatic) and ? (unidentified tissue source for this LSC). Stage of cDNAs PCRd is indicated by Z# for zygotic and stage of development, LSC for liquid suspension culture, S# for somatic and stage of development, and Late for late stage somatics. Arrow indicates PCR product (upper band) lower band represent unused primers



VI. CONCLUSIONS

A number of expression markers for pine embryo development have been identified and analyzed. Several cDNA clones of mRNAs which are present early in embryo development have been cloned. Some have been sequenced and a number of interesting similarities to characterized genes from other organisms have been noted. Northern analysis using one of the cDNAs as probe with RNA from Liquid Suspension Culture and Stage 9 Somatic Embryos, confirms differential expression. RT-PCR experiments show differential expression throughout embryo development and differences between somatic and zygotic embryos. These experiments are being repeated and extended.

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COMBINED EFFECTS OF ACTIVATED CARBON AND pH ON IONIC COMPOSITION AND 2,4-D AVAILABILITY IN A TISSUE CULTURE MEDIUM

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ABSTRACT

Activated carbon (AC) is added to tissue culture medium, often giving positive results. Two different activated carbons have been found to give different success rates in tissue cultures. The two carbons have been characterized using several different techniques with the goal of correlating character with impact on medium composition. Ash %, point of zero charge (PZC), and apparent surface area, showed the greatest differences. Medium pH was found to vary with AC type, and preparation technique. The combination of AC and medium pH (ranging from pH 4.8 to pH 6.8) could significantly alter the ionic composition of the medium, resulting in decreased Cu (-90%), Zn (-50%), Fe (-50%) and Mn (-60%) and increased Mg (+50%). The sorption of 2,4-D varied with apparent surface area (BET) and system pH: the combination of factors resulting in nearly 50% differences in sorption capacity. These findings may help to explain some of the contradiction in the literature regarding the benefits of including AC in tissue culture medium.

INTRODUCTION

The addition of activated carbon (AC) to plant tissue culture medium has been shown to benefit many different tissue culture systems, including algal cultures (1), anther cultures (2, 3), date palm cultures (4), and Douglas-fir and other conifer cultures (5). Several different hypotheses have been advanced to explain the observed benefits: adsorption of growth inhibitors (toxic metabolites) and prevention of unwanted callus growth (6), adsorption of breakdown components of sucrose (7), removal of excess hormone (2,4-D) (8) and release of substance (9) and the sorption of mineral nutrients (10, 11).

Anther culture research has shown that success may vary with the source of activated carbon (12). Work with Douglas-fir somatic embryogenesis at IPST (unpublished results) has revealed that acid-washed activated carbon performs differently from non-acid washed activated

carbon. Further work at IPST using a Norway spruce somatic embryogenesis system has shown that success may also vary with AC production lots.

Research at IPST has shown that the presence of AC in media used for initiation of loblolly pine results in a 50% decrease in available zinc and a 90% decrease in available copper (13). The sorption of 2,4-D onto a single activated carbon was modeled, allowing predictions of available 2,4-D based on initial relative concentrations of 2,4-D and AC (13).

The physical and chemical properties of AC derive from its extensive surface area, which may include a high percentage of micropores (pores less than 2 nm) and its surface chemistry, which is primarily determined by functional groups containing oxygen. Activated carbon is produced through oxidation of pyrolyzed material, which is subsequently pulverized. This oxidation may be achieved with acids, typically nitric or phosphoric, or using high temperature treatments under an oxidizing atmosphere, commonly steam or CO₂. The surface may be either acidic or basic (14).

In this on-going study we seek to correlate the physical/chemical characteristics of activated carbon with the impact of AC on the medium composition, and subsequently, its impact on culture success. This paper reports results for two different activated carbons for the sorption of 2,4-dichlorophenoxy acetic acid (2,4-D) from aqueous solution as well as AC impact on cation concentrations in an initiation medium (Norway spruce).

EXPERIMENTAL

Characterization of Activated Carbon

Activated carbon was supplied by Sigma as untreated powder (C-5260), designated "N" type, and acid-washed tissue culture tested (C-9157), designated "T" type. Two production lots, designated "1" and "2", for each type were characterized. For each characterization, unless stated otherwise, at least two replications were performed.

Ash % was determined on dry AC (dried using a vacuum oven at 120°C overnight) through thermo-gravimetric analysis. The point of zero charge, PZC, was approximated through mass titration (15) which simply involved adding an increasing mass of AC to a given mass of water until some limiting pH value was approached. Apparent surface area (BET) was determined through nitrogen sorption (Micromeretics Flowsorb II 2300).

2,4-D Sorption

Experiments were performed using sealed 40mL glass vials or 200mL plastic bottles. To reach pH targets, acid solution (0.01N or 0.1N HCl) was first added to AC and allowed to equilibrate. The proper amount of acid had been previously determined through titrations and trial and error. Phosphate buffer, adjusted to the final pH target, was added such that the final diluted concentration was approximately 0.0125M. Stock solution of 2,4-D (1g/L) was added to give a final concentration of 200mg/L (200ppm). All aqueous phases were mixed using degassed, deionized water. All vessels were shaken for the duration of the experiment. Experiments were performed in duplicate.

Following sorption, typically longer than five days, samples (10mL) were taken from the vessels and passed through a syringe filter (Gelman Acrodisc 0.2 μ m, HT Tuffryn® membrane). The first 1.5 mL were discarded. The 2,4-D concentration was measured at 284nm (Beckman DU 640 Spectrophotometer).

Cation Analysis

Ion analyses were performed on complete and partial media. Experiments were performed in duplicate. The medium consisted of a liquid version of the Institute of Paper Chemistry Norway spruce initiation medium (16), with 2,4-D (2ppm) substituted for NAA, and BAP (1ppm) as specified previously. When activated carbon was present (1.25 g/L), the hormone levels were elevated to 90ppm BAP and 100ppm 2,4-D. Media were typically allowed to equilibrate three days at room temperature (21°C) before being analyzed. Samples (approximately 7.5mL) were collected as per the 2,4-D procedure. Two drops of concentrated nitric acid were added to each sample. The samples were then analyzed for cations using inductively coupled plasma atomic emission spectroscopy (ICP-AES, Perkin Elmer Optima 3000 DV). Each ICP measurement was replicated three times.

RESULTS

Characterization of AC

Mean values for the characterization results are summarized in Table 1. The ash percentage varied significantly between AC types, with N1 and N2 containing more than twice as much ash percent as T1 or T2. Significant variation in ash % was evident between batches 1 and 2 for the N-type AC. Analysis for trace metals (SEM-EDS) failed to detect significant differences in atomic species present in the ash for the different AC samples: the release of toxic metals does not appear to be a concern when using these AC's.

Table 1. Summary AC Character

	Ash %	PZC, pH	BET m ² /g
T1	2.8	8.5	1050
T2	2.6	10	940
N1	5.8	10.7	560
N2	8.7	11	710

The point of zero charge (PZC) characterization gives the pH at which the sum of the charges on a particle suspension is equal to zero. This measurement differs from the isoelectric point (IEP) in that the isoelectric point is determined from moving particles: the internal surface is not important to IEP determinations. The PZC data indicate that the T1 and T2 are less basic than N1 and N2. Since the pH scale is logarithmic, a pH unit of difference is quite significant at pH 10. It can be concluded that the carbon types are significantly different with respect to PZC. The difference between T1 and T2 indicates that production lots may vary significantly.

The apparent surface area ranged from 560 m²/g to 1050 m²/g. Comparing the average of T1 and T2 to that for N1 and N2, it may be seen that the difference due to carbon type was approximately 350m²/g. The greatest difference was between T1 and N1. These were the two carbons which have been used in tissue culture media at IPST. The between-batch variation, i.e. T1 vs. T2, and N1 vs. N2, appears to be significant.

Culture Medium pH

As a result of the AC characterization, experiments were conducted in which media were mixed, allowed to equilibrate two days and pH measurements made. The two day period was representative of what might occur in actual lab practice.

According to our procedure at IPST, the medium pH was adjusted after adding AC, prior to autoclaving. In this case, the target pH was 5.8. As can be seen from Figure 1, the amount of time allowed for pH adjustment influenced the pH of the medium after two days. Referring to the figure, the x-axis is labeled according to the amount of time allowed to adjust pH after addition of AC to the medium. For the media labeled, "0 min", the pH was adjusted prior to AC addition with no further adjustment after AC addition. As more time was allowed for pH adjustment after AC addition, the closer was the final pH to the target. The tendency, however, was for the pH to drift in the basic direction when AC was present.

After two days, N1 gave higher pH for each treatment, approaching pH 6.8 when no time was allowed for pH adjustment. Past experience has shown that the pH of the

medium just after autoclaving is typically below 5.8, about 5.6 for T-type and above 6.5 for N-type. Titrations with acid have revealed that ten times as much volume is required to reach a given pH point for N-type vs. T-type carbons. Also, it has been found that pH drift after the titration end point has been reached is much more significant for the N-type carbons: it is much more difficult to obtain pH 5.8 with the N-type carbons.

For media exposed to a growing Norway spruce culture, the medium pH drops to about 4.8 over a period of days or weeks. This phenomenon has been observed for media with T-type AC and for media without AC. When N-type carbons have been used, this drop tends to be much more gradual and doesn't dip below pH 5.

Therefore, the pH range of interest for this research is from pH 4.8 to pH 6.8.

Sorption of 2,4-D pH effect.

Data were collected at room temperature (21°C) for sorption of 2,4-D (200mg/L, 200mL volume, phosphate buffer) onto AC (0.125g/L) over the pH range of 4.8 to 6.8. These relative concentrations were chosen after initial experiments revealed that 2,4-D was being depleted at ten to twenty times more than what had been expected based on previous literature (17). It was found that equilibrium was reached within two days but typically more than five days were allowed before measurements were taken. These data have been normalized to sorption capacity at pH 6.8 and are presented in Figure 2. It can be seen that there was a trend for both carbons towards greater sorption of 2,4-D as the pH declined from 6.8. In fact, at pH 4.8 vs. 6.8 the sorption capacity has increased by more than 40% for both carbons.

Apparent surface area.

Using the same relative concentrations of 2,4-D and AC as the previous experiments and adjusting pH to approximately 6.8 for each solution, sorption data were generated for each of the four activated carbons. These data were then plotted as a function of apparent surface area in Figure 3.

The two samples per AC type show a good deal of spread in their sorption capacity, indicating a high level of variability in the material. The mean trend is that sorption capacity for 2,4-D increases with apparent surface area. Comparing mean values for T1 and N1, the difference is about one third more sorption onto T1.

Ion experiments.

Equilibrium conditions were reached within a day but typically two days were allowed before samples were analyzed. Data were initially produced for thirty ions. However, with the exception of silica, neither N1 nor T1 were releasing measurable quantities of non-nutrient ions. The media at pH 4.8 were visibly clearer than media at higher pH. After autoclaving, a precipitate was visible in media with pH above 5.8. The precipitate could be avoided by omitting the macronutrients from the medium.

Figures 4 and 5 present data for copper and zinc, respectively. The concentration of each ion is depicted as a function of medium pH. Media without carbon are designated "Con" short for "control" and were otherwise identical to media which included AC. Across the top of each figure a line is drawn defining the concentration of ion calculated to be present based on the medium "recipe".

From the data in Figure 4 it can be seen that more than 90% of the copper was depleted compared to the control. The control levels were in good agreement with the calculated values. There appeared to be a slight increase in copper availability as pH increased. The data for the two different AC's showed no difference.

Figure 5 shows that the control responded to changes in pH. About 50% of the zinc was depleted in response to a pH increase from pH 5.8 to pH 6.75, without AC present. The presence of AC resulted in reduced availability of zinc compared to the control across the pH range of 4.8 to 6.75. The scatter in the data prevented conclusions as to whether there was an effect due to AC type.

Figure 6 presents data for iron. All of the media were low in iron relative to the calculated amount, across the pH range studied. This phenomenon has been observed previously and attributed to the precipitation of phosphate and Fe-EDTA (18). Relative to the amount of iron present at pH 4.8, about 50% was depleted as pH increased to 6.8. The control sample showed higher precipitation than the activated carbon samples.

Data for magnesium are depicted in Figure 7. Only the media with N1 showed significant deviation from the calculated value: N1 added about 50% more magnesium relative to the calculated value. A pH effect was not observed in the control or for media containing T1.

Manganese showed a very large pH-dependent drop (Figure 8); more than 60% was depleted as pH increased from 5.8 to 6.8. This drop was independent of the presence of AC.

Molybdate was reduced more than 50% across the pH range, independent of AC. Cobalt was reduced to levels near zero and its importance to the medium needs further investigation.

CONCLUSIONS

The T type activated carbons are different physically and chemically from the N-type, with at least 50% more apparent surface area, half as much ash % and a PZC which is 1 pH unit lower than the N-type carbons. There are also differences between the production lots within either type, most notably for BET surface area.

The pH of the medium may have a profound impact on the medium composition. Medium pH is influenced by AC type and how much time is allowed for pH adjustment prior to autoclaving the medium.

The sorption of 2,4-D onto AC appears to be a function of the apparent surface area and the pH of the medium. The combination of effects when comparing media with T1 to media with N1 results in about 40% more 2,4-D sorption capacity for T1 (over 40% more 2,4-D available in media with N-type AC).

Several ions showed increased availability as the pH of the medium declined from 6.8 to 4.8: P (+33%), Mn (+200%), Fe (+140%), Zn (+190%).

Including AC in the medium results in the increased availability of several ions. A greater effect was observed for N1, with increased Si (+30%), Mg (+50%), Ca (+15%), and Al (+300% approx.).

Only two ions appeared to be sorbed onto AC : Cu (-90%) and Zn (-50%). When AC was present, the availability of Cu increased as pH increased.

Figure 9 summarizes a few of the changes in medium composition which would result from substitution of N1 for T1. The data have been normalized against a medium containing T1 at pH 5.8.

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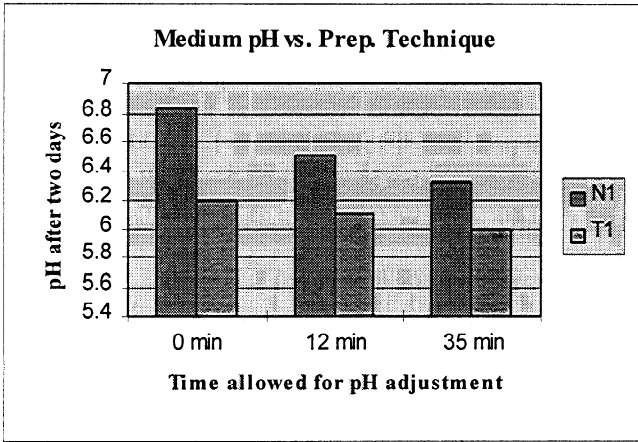


Figure 1. Medium pH as a function of the time allowed for pH adjustment after the addition of activated carbon (1.25 g/L). Media have been adjusted to pH 5.8 and then autoclaved and stored for two days prior to measurement.

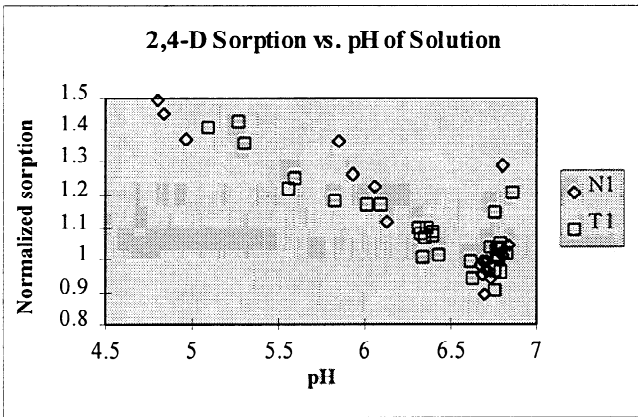


Figure 2. Sorption of 2,4-D (initial conc. 200ppm) onto activated carbon (0.125 g/L) at room temperature from buffered aqueous solution. The data for each carbon type were normalized to their respective mean values at pH 6.8.

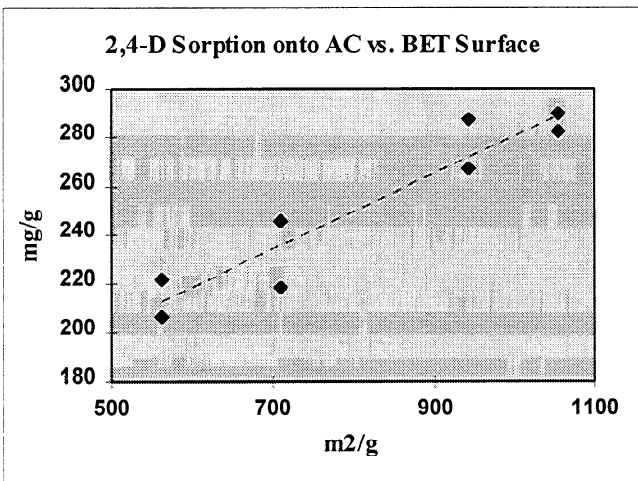


Figure 3. Sorption capacity for 2,4-D as a function of BET surface area of activated carbon. The experimental conditions were: 2,4-D, 200 ppm; AC, 0.125 g/L; pH 6.8; room temp. (21°C). The range in apparent surface area for the four different activated carbons is presented in Table 1.

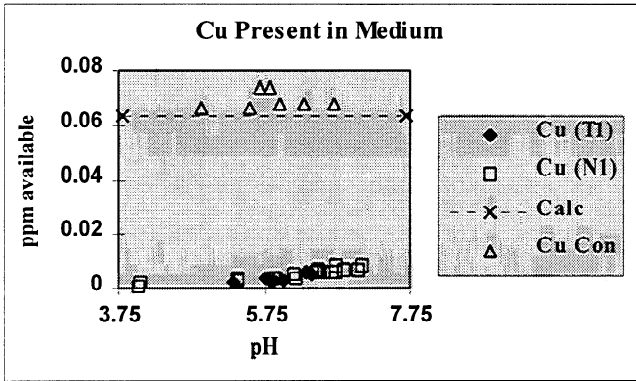


Figure 4. Copper availability vs. medium pH. Data were produced using ICP. Media were complete, including 2,4-D at 100ppm and BAP at 90ppm. Experiments were conducted with one production lot of each AC type, designated T1 and N1. "Calc" designates the amount of ion expected to be present. The control, designated "Con", contained no AC but was otherwise identical to media with AC.

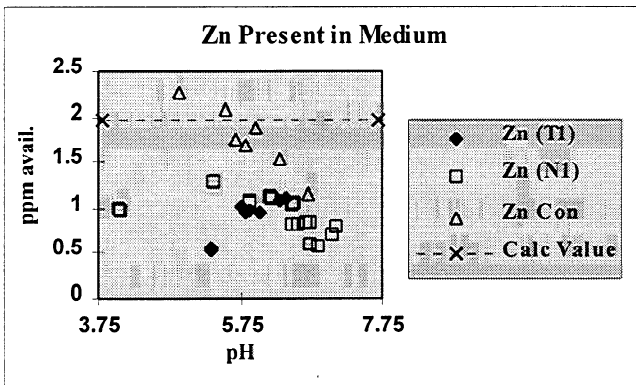


Figure 5. Zinc availability vs. medium pH. See caption for Figure 4.

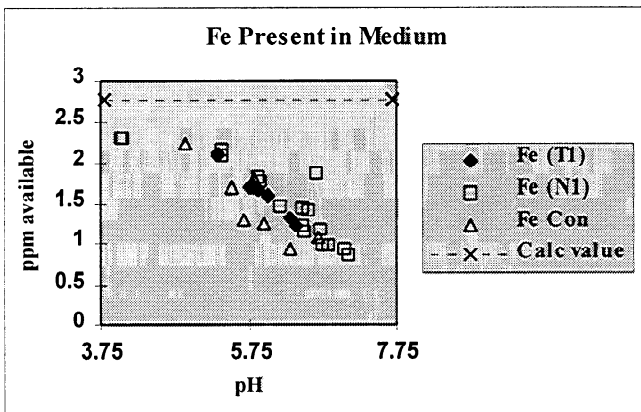


Figure 6. Iron availability vs. medium pH. See caption for Figure 4.

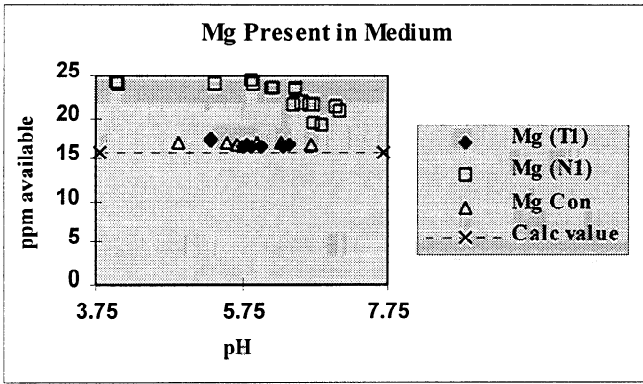


Figure 7. Magnesium availability vs. medium pH. Data were produced using ICP. Media were complete, including 2,4-D at 100ppm and BAP at 90ppm. Experiments were conducted with one production lot for each AC, designated T1 and N1. “Calc” designates the amount of ion expected to be present. The control, designated “Con”, contained no AC but was otherwise identical to media without AC.

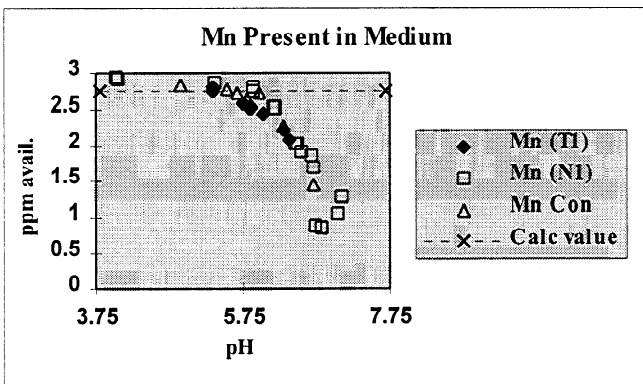


Figure 8. Manganese availability vs. medium pH. See caption for Figure 7.

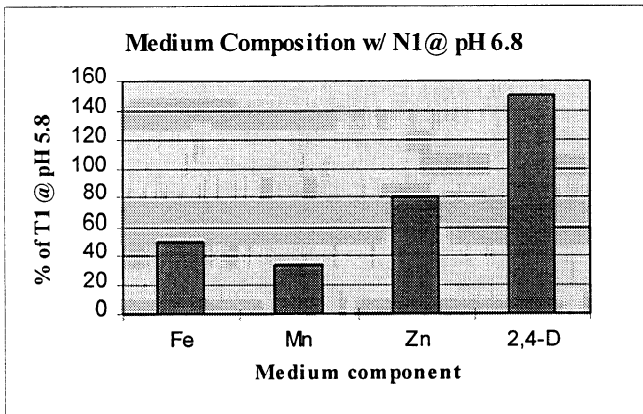


Figure 9. Impact on medium composition of substituting N1 for T1, including pH effects. Data have been normalized to expected medium composition when T1 is present at pH 5.8

HARDWOODS

**DUES FUNDED RESEARCH CONSORTIUM
1997-1998**

**MASS CLONAL PROPAGATION OF GENETICALLY
IMPROVED HARDWOODS**

**Status Report for
Project F011**

**Gary Peter
John Cairney
Gerald Pullman
Cielo Castillo**

March 26-27, 1998

DUES-FUNDED PROJECT SUMMARY
1997-1998

Project Title:	Improved Stem Growth Rates and Fiber Properties: Fundamental Biological Mechanisms
Project Code:	HRDWD
Project Number:	F011
PAC:	Forest Biology
Division:	Chemical and Biological Sciences
Project Staff	
Faculty/Senior Staff:	Gary Peter, John Cairney, John MacKay, Jerry Pullman
Staff:	Cielo Castillo
FY 97-98 Budget:	130,494
Allocated as Matching Funds:	33,505 (25.7%)
Time Allocation	
Faculty/Senior Staff:	0.4
Support:	1.0
Supporting Research	
M.S. Students:	Douglas Benton
Ph.D. Students:	none
External:	Louis Destefano, Haubin Meng (postdoctoral fellows)

RESEARCH LINE/ROADMAP: (New from Spring 1998)

<i>Area</i>	Improved Forest Productivity
<i>Research Line</i>	Develop fibers with properties similar to or better than Northern softwood and Eucalyptus which can be grown in most regions of North America.
<i>Road Map</i>	Develop fundamental understanding of secondary wall differentiation and stem growth

PROJECT OBJECTIVES:

This project has three broad objectives: 1) increase the growth rate of the stem, 2) improve fiber properties for value added paper products and 3) improve the processing characteristics of wood to decrease environmental impacts while increasing fiber yield and quality. Since our understanding of the mechanisms that regulate cambial cell growth rate, xylem fiber and secondary cell wall properties is so limited, to accomplish these goals a more fundamental understanding of the biochemical, cellular and molecular genetics in each of these areas must be obtained first. Although we would like to begin in parallel a coordinated effort in each of these areas, the limited funds in this project dictate that we work on them in sequential order based upon prioritization's. Our efforts toward this project will accelerate once additional moneys are obtained



PROJECT BACKGROUND:

The objectives of this project were changed recently. Previously this project was focused on developing efficient transformation methods for commercially important hardwood species. To align the objectives of this project better with member company needs the F011 project has taken a new direction. In the Spring of 1997, a new global plan for this project was submitted to the PAC for review. A unanimous decision was reached for this project to pursue more fundamental research into the biochemical, cellular and molecular genetic mechanisms that control stem growth rates and fiber cell properties, including, cell size, shape, secondary wall composition and organization. In addition, there was general agreement that this project should no longer focus exclusively on hardwood species, but rather include model dicot systems, hardwood as well as softwood species. Although since hardwoods can be genetically transformed initial bioengineering experiments will be carried out with *Populus deltoides*. The current focus of this project is to elucidate the mechanisms that control fiber and cambial cell properties.

Three Year Research Plan for Mass Clonal Propagation of Genetically Improved and Engineered Hardwoods 1997-2000

Gary Peter, John Cairney, Gerald Pullman

Executive Summary: This plan represents our effort to develop an integrated program that addresses research areas of high value to member companies both now and in the future. The proposal attempts to balance the unique strengths of IPST faculty as a whole with our specialized expertise in plant tissue culture, gene regulation, gene identification and xylem tracheary element differentiation to achieve important goals defined both by the research lines of IPST and by member companies. The plan proposes 80% of the work on cottonwood and 20% on other species as models. This overly ambitious plan was written to focus discussions on which are the most important directions and goals for the hardwood program at IPST.

- Objectives:**
1. To increase the efficiency of transformation methods used for commercially important cottonwood genotypes from the west and the southeast
 2. To identify and characterize robust gene regulatory sequences that can be used for stable gene expression in forest trees independent of transformation event, tissue type or cell type
 3. To isolate and characterize gene regulatory sequences that direct expression in cambial meristem cells and differentiating xylem cell types leading toward the regulation and control of valuable fiber cell properties
 4. To isolate genes that regulate valuable fiber properties e.g., secondary cell wall thickness, cellulose microfibril angle

Recent History & Accomplishments: The hardwood program focused on developing methods to transform cottonwood. IPST researchers lead by Ron Dinus were one of the first to transform *Populus deltoides* or Eastern cottonwood. An organogenic regeneration method using stem tissue was developed for a model clone, C175, which originated in Minnesota and was obtained from the



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(For Member Company's Internal Use Only)

University of Nebraska. This genotype was transformed with the GUS reporter gene using *Agrobacterium tumefaciens*. Transformed plants resistant to kanamycin were regenerated. The introduction of foreign DNA was confirmed both by positive GUS staining and genomic DNA gel blot analysis.

Work towards understanding gene regulation of both hardwoods and softwoods is being led by John Cairney. A number of drought induced genes were isolated from the desert shrub *Atriplex canescens*. The structure and function of these genes and the proteins they encode are being characterized. Work towards producing sterile and early flowering tree species has begun with funds from the Georgia Consortium for Technological Competitiveness in Pulp and Paper. The approach being followed is to isolate floral meristem and organ regulatory genes and to inhibit their expression with antisense or dominant negative protein strategies. The focus of our research is to isolate floral meristem and organ regulatory genes from *Pinus taeda*. Such clones will be a resource which can be explored through collaboration with other groups and will provide the basis for an externally funded project. We intend to leverage these state funds to improve our hardwood transformation efforts.

Future Visions: The forest biology team visualizes a state of the art hardwood program which will use commercial cottonwood lines to focus on 1) improving transformation frequency, 2) understanding gene regulation so researchers can add foreign genes and cause them to function where and when desired, and 3) to target fiber modification as value added changes. It is expected that this program will not only result in improved techniques but also create valuable cottonwood clones with modified growth and fiber properties.

I. Vegetative Propagation

Goal: To develop efficient in vitro regeneration methods that are useful for genetic transformation of Populus species

Year 1- Initiate *in vitro* regeneration experiments with commercially relevant *Populus* clones from the northwest and southeast

1. Identify and select cottonwood clones
 - a) Select one model *Populus* hybrid clone from the northwest
 - b) Select 1-2 *Populus deltoides* clones that represent elite genotypes for the southeast
2. Acquire cuttings and seeds, initiate plants for explant material
3. Develop culture conditions that stimulate organogenesis/regeneration in new clones
 - a) Test standard methods developed with C175
 - b) Adapt method for commercially relevant *P. deltoides* genotypes. Test explant type, media composition, hormone concentrations....
4. Initiate transformation studies with *Populus* hybrid clone from the northwest (see section II for details)

Year 2- Initiate transformation studies with *P. deltoides*

1. Continue to improve regeneration systems for *P. deltoides* clones
2. Regeneration system suitable for transformation target



Year 3- Acquire additional elite cottonwood clones and establish robust regeneration methods for new clones that show promise from breeding studies

1. Establish connections with cottonwood breeding programs
2. Obtain new cottonwood clones
3. Initiate regeneration tests with more valuable cottonwood clones

II. Genetic Transformation of *Populus* Species

Goals: To develop vectors and methods useful for efficient selection of transformed Populus tissues

Year 1- Initiate transformation of *Populus* hybrids

1. Begin transformation experiments with *Populus* hybrid testing various drug resistance genes and selection schemes, e.g., neomycin phosphotransferase, phosphinotricin, acetolactate synthase, ... (see section III for details)
2. Initiate cell suspension of cottonwood cell line for transient assay work

Year 2- Initiate transformation of *P. deltoides*

1. Transform *P. deltoides* with *A. tumefaciens* and test efficacy of promoter selectable marker combinations in transformation procedure, begin to establish rapid and efficient methods
2. Transformation target for elite clone: 5-10% success

Year 3- Improve transformation procedures with *Populus* clones and if necessary expand range of genotypes in the program

III. Gene Regulation

Goals: To develop techniques for use in cottonwood improvement through genetic engineering. Develop nonproprietary vectors for stable, constitutive, high level expression independent of tissue and transformation event. Isolate sequences useful for targeted expression within the cells of the cambial meristem and xylem cell types.

Year 1- Construct nonproprietary sequences for stable high level expression of transgenes useful for transformation studies and agronomic trait introduction

1. Isolate strong, constitutively expressed gene from *Populus* cells to drive selectable marker gene expression that will not be proprietary like CaMV 35S-neomycin phosphotransferase
 - a) Isolate cDNA expressed strongly in proliferating tissues; e.g., ribosomal proteins, translation factors, cell division cycle genes, actin, tubulin, prove expression pattern and level by RNA gel blots and *in situ* hybridization
 - b) Isolate gene and define start site of mRNA
2. Construct expression cassette to test promoter expression with green fluorescent protein (GFP) or b-glucuronidase (GUS)



3. Identify sequences that may affect stability and translatability of foreign mRNAs.
 - a) Continue investigating the functionality of AU rich sequence identified from drought induced gene in stabilizing foreign mRNAs in transient assays with cottonwood cell lines and model plants
 - b) Determine whether viral translational enhancers improve the translatability of foreign mRNA in transient assays in cottonwood cell lines and transgenic cottonwood trees

Year 2- Isolate cell type specific cDNAs from the secondary vascular tissues of cottonwood to ultimately obtain a panel of regulatory sequences that confer cell type specific expression patterns in secondary xylem tissues

1. Construct cDNA libraries to secondary vascular tissues of cottonwood at various points in the growth season
2. Begin isolating cDNAs that are expressed specifically in cambial meristem cells, xylem fibers, xylem tracheary elements, vessel elements, ray cells, parenchyma cells....
3. Begin creating transgenic cottonwood trees that test the functionality of high level expression elements

Year 3- Integrate understanding of transcriptional and post-transcriptional gene regulation to create improved plant transformation vectors for optimal tissue specific expression.

1. Evaluate these vectors in transgenic cottonwood and model plants
2. Evaluate transgenic cottonwood for quantitative effect of sequence elements in mRNA stability and protein expression

IV. Isolate Genes that Regulate the Rate of Cell Division in the Cambial Meristem, Xylem Differentiation and Fiber Cell Properties

Goals: Isolate genes that can stimulate the rate of cell divisions in the cambial meristem. Identify genes that regulate xylem tracheary element cell size and secondary cell wall properties such as wall thickness and cellulose microfibril angle.

Year 1- Explore approaches for creating transgenic cottonwood with improved cambial growth and fiber properties and characterize fiber properties of short rotation cottonwood.

1. Investigate the feasibility of using conserved sets of genes that when overexpressed in cottonwood are predicted to stimulate the rate of cambial cell divisions, promote xylem tracheary elements or fiber differentiation, stimulate xylem cell elongation...
 - a) Targeted genes might include cell division cycle genes, hormone biosynthetic genes, hormone response genes
2. Continue development and use of *Z. elegans* as a model system for isolating and elucidating the mechanisms that regulate tracheary element differentiation and fiber properties



- a) Develop culture methods, transient assays and microscopic analyses to enhance utility of the *Z. elegans* model system
 - b) Analyze rac GTPase-proteins investigating their role in regulating cytoskeletal organization and directing cellulose synthesis in *Z. elegans*
 - c) Isolate cellulose synthase gene(s) from *Z. elegans* tracheary elements and develop tagged version of cellulose synthase for identifying other subunits, subcellular/membrane localization's, interactions with microtubules.
3. Isolate cellulose synthase gene(s) from cottonwood cambium and differentiating xylem cells
 4. Develop links with experts at IPST to measure wood and fiber characteristics of short rotation cottonwood

Year 2- Utilize *Z. elegans* for testing the effect of overproducing cellulose synthase subunits during tracheary element differentiation and begin investigating the mechanism by which microtubules interact with the cellulose synthase complex. Continue with genetic engineering strategy for enhancing growth of the cambial meristem in cottonwood

1. Test if overexpression of the cellulose synthase catalytic subunit in differentiating xylem tracheary elements of *Z. elegans* increases cellulose production
2. Create cottonwood cell line expressing epitope tagged cellulose synthase and immunoprecipitate cellulose synthase complex from purified plasma membranes
 - a) Initiate biochemical analyses of cellulose synthase complex
 - b) Investigating cellulose synthase interaction with cytoskeletal components
 - c) Look for presence of rac GTPases in cellulose synthase complex
3. Isolate cDNAs and their corresponding genes which are expressed in specific xylem cell types
4. Transform cottonwood with gene that will stimulate the xylem production or cell division rates of cambial cells

Year 3- Implement strategies proven with *Z. elegans* to improve fiber properties by creating transgenic cottonwood plants. Analyze the properties of the cambium and secondary xylem in transgenic cottonwood

1. Transform cottonwood gene(s) to enhance cellulose production and/or cellulose microfibril angle
2. Analyze transgenic cottonwood created in year 2



SUMMARY OF RESULTS:

1. We have reestablished the tissue culture methods (shoot regeneration from leaves and internodes) and are currently testing the transformation methods with *Populus deltoides* C175 that were previously developed in this program to use for future transformation experiments.
2. A number of vectors were constructed for transient transformation experiments with *Z. elegans*
3. We have obtained a high quality cDNA library constructed with RNA isolated from Aspen cambial and differentiating xylem cells from Lin Ge a postdoctoral fellow in our group. This library was amplified and DNA was extracted to serve as a source of messages for gene isolations by PCR.
4. We have isolated the cambial region/differentiating xylem tissue from loblolly pine stems for RNA isolation and cDNA library construction.
5. We have cloned the conserved region from celA of *Arabidopsis thaliana*. CelA is the catalytic subunit of the cellulose synthase complex.
6. We have assembled an alignment for the DNA and protein sequences of all known celA like genes. Using this alignment we have designed degenerate primers for celA isolation from *Z. elegans*, *Populus* and or *Pinus taeda*.
7. We have discovered a simple and rapid microscopic method for rapidly measuring microfibril angle in loblolly pine tracheids.
8. We have constructed a DNA and protein alignments for all three classes of cyclins from plants. Degenerate primers have been designed from the protein alignment.

GOALS FOR FY 97-98:

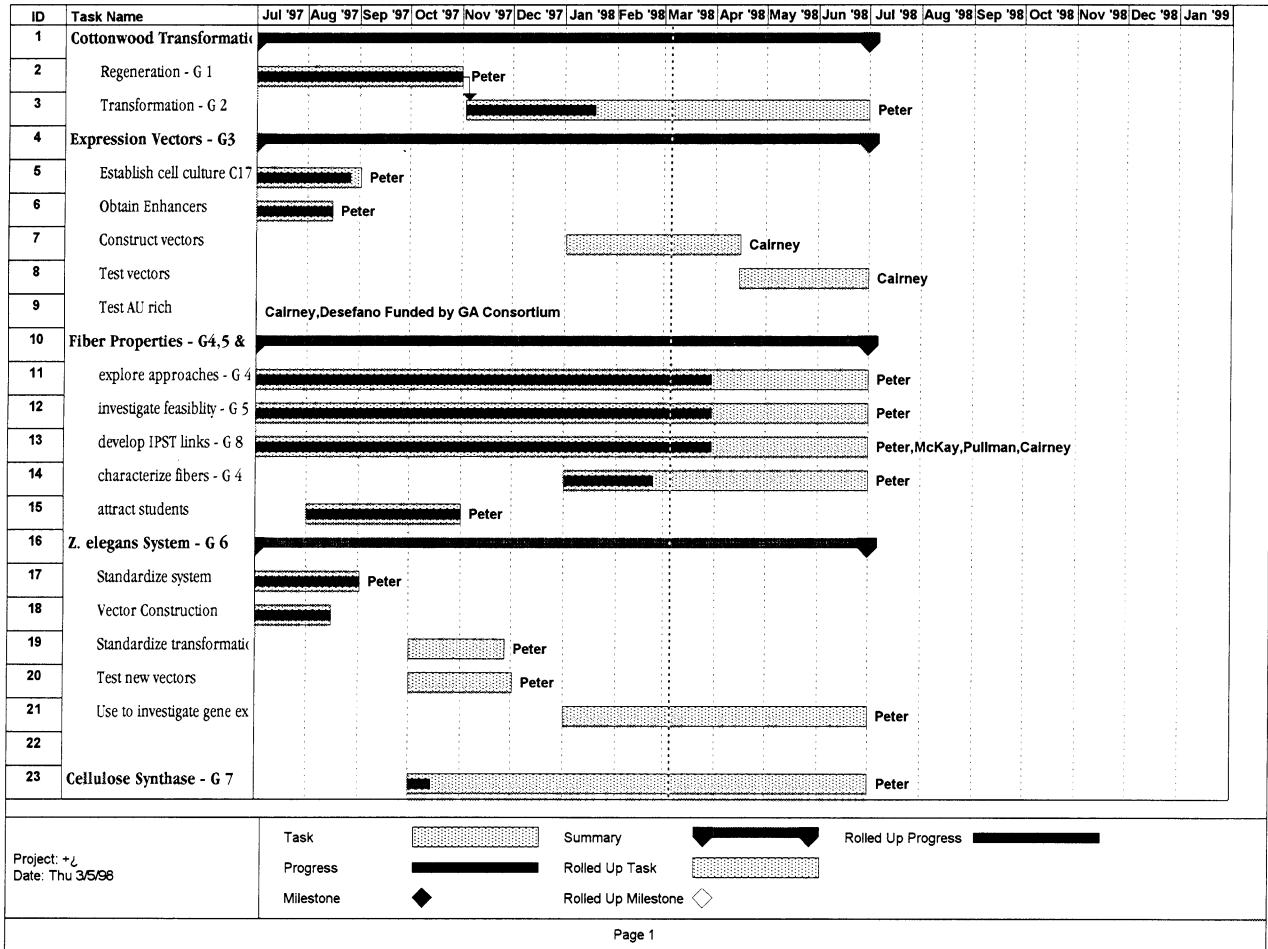
1. Initiate *in vitro* regeneration experiments with commercially relevant *Populus* clones from the northwest and southeast. (No work done based on feedback from PAC)
2. Initiate transformation of *Populus* hybrids and *Populus deltoides* (Procedures being restandardized with *P. deltoides* C175)
3. Construct nonproprietary sequences for stable, high level expression of transgenes useful for transformation studies.
Evaluate the effect of an AU rich sequence on the stability / translation of a mRNA. (Work is being done with external funds obtained by J. Cairney and J. Pullman from Georgia Consortium)
Test usefulness of viral translational enhancers in transient assays. (Enhancers obtained)
4. Explore approaches for creating transgenic cottonwood with improved cambial growth and fiber properties and characterize fiber properties of short rotation cottonwood. (A continual pursuit of this goal will always be carried out)
5. Investigate the feasibility of using conserved sets of genes that when overexpressed in cottonwood are predicted to stimulate the rate of cambial cell divisions, promote xylem tracheary elements or fiber differentiation, stimulate xylem cell elongation... (This work is feasible)
6. Continue development and use of *Z. elegans* as a model system for isolating and elucidating the mechanisms that regulate tracheary element differentiation and fiber properties. (Constructed vectors to enhance transient transformation funded by Seed grant to G. Peter)
7. Isolate cellulose synthase gene(s) from cottonwood cambium and differentiating xylem cells. (Work toward this goal is in progress)
8. Develop links with experts at IPST to measure wood and fiber characteristics of short rotation cottonwood. (Have established multiple links and collaborative relationships; have developed a simple and rapid method for measuring microfibril angle in loblolly pine)



DELIVERABLES:

Report describing results and research progress in the fall of 1997.
 Annual report describing results and research progress in the spring 1998.

SCHEDULE (Attach Timeline):



FO11 MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED HARDWOODS

Progress Toward Goals

Gary Peter
 John Cairney
 Gerald Pullman
 Cielo Castillo

For most of the work we have been laying a foundation for our work in the area of elucidating the mechanisms that regulate in stem growth rate and fiber properties.

1. Initiate transformation of *Populus* hybrids and *Populus deltoides*.

We have been restandardizing tissue culture and transformation procedures with *P. deltoides* C175. We initiated a transformation experiment testing two strains of *Agrobacterium tumefaciens* both containing the green fluorescent protein.

2. Construct nonproprietary sequences for stable, high level expression of transgenes useful for transformation studies.

A) Evaluate the effect of an AU rich sequence on the stability / translation of a mRNA.

Work is being done with external funds obtained from Georgia Consortium by J. Cairney and J. Pullman

B) Test usefulness of viral translational enhancers in transient assays.

Enhancers were obtained, but no further work was done since this isn't a priority.

3. Explore approaches for creating transgenic cottonwood with improved cambial growth and fiber properties and characterize fiber properties of short rotation cottonwood.
4. Investigate the feasibility of using conserved sets of genes that when overexpressed in cottonwood are predicted to stimulate the rate of cambial cell divisions, promote xylem tracheary elements or fiber differentiation, stimulate xylem cell elongation...

We have written proposals to external funding agencies for selectively increasing stem growth rates by ectopic overexpression of cyclin cDNAs in the cambial meristem cells. We have constructed DNA and protein sequence alignments for all cyclins in plants and designed degenerate primers. We have obtained a cDNA library constructed from mRNA isolated from the cambium/xylem of Aspen. We have isolated tissue from the cambium/xylem and cambium/phloem of loblolly pine.



5. Continue development and use of *Z. elegans* as a model system for isolating and elucidating the mechanisms that regulate tracheary element differentiation and fiber properties.

We constructed two separate vectors to enhance our transient transformation of *Z. elegans*. This work was funded by a IPST/GIT Seed grant to G. Peter)

6. Isolate cellulose synthase gene(s) from cottonwood cambium and differentiating xylem cells.

We have created DNA and protein sequence alignments of all known celA genes and designed degenerate primers. We have cloned the highly conserved domain from one of the Arabidopsis celA genes.

7. Develop links with experts at IPST to measure wood and fiber characteristics of short rotation cottonwood.

We have established multiple links and collaborative relationships; with other faculty members in wood chemistry and fiber and paper physics. We have developed a simple and rapid method for measuring microfibril angle in loblolly pine trachieds.



F011: MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED HARDWOODS

Establishing and Improving the *Zinnia elegans* Tracheary Element Differentiation System at IPST

Gary Peter
Karen Floyd
Chris Ricker

Introduction:

Zinnia elegans is a model tissue culture system that can be used to study cellular and molecular mechanisms that control tracheary element (TE) differentiation. This culture system was developed in the early 1980's (H. Fukuda and A. Komamine, 1980 Plant Physiol. 65: 57-60). In this system primary leaves from 10-14 day old seedlings are harvested and surface sterilized. Individual cells are mechanically isolated by grinding and cultured in defined media. In the presence of auxin and cytokinin ~50% of the isolated mesophyll cells transdifferentiate into TEs within 72 hours. The properties that make this a valuable model system include its hormone inducibility, synchrony, and single celled nature. This system has been used to elucidate aspects of hormone signal transduction in TE differentiation, cytoskeletal control of secondary cell wall formation, biochemical analyses of lignin biosynthesis, identification of genes expressed in differentiating TEs, and programmed cell death (H. Fukuda, 1996 Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 299-325; H. Fukuda, 1997 Plant Cell 9 (7)).

One of the major technical challenges to improve this system for investigating regulatory mechanisms was to develop a robust transient genetic transformation method. Transformation can then be used to identify gene regulatory sequences, help elucidate the function of unknown genes expressed in TEs, and to rapidly test strategies for altering TE formation. Previously at University of California Berkeley, I developed a transient transformation assay for *Z. elegans* utilizing *Agrobacterium tumefaciens* (G. Peter, unpublished).

We are now standardizing this transformation assay to the growth conditions used here at IPST. We are also designing, constructing, and testing gene expression vectors to use in transient assay experiments that are meant to elucidate the function of novel gene products in TE differentiation and secondary wall formation with the *Z. elegans* model system.

Critical Parameters for Establishing *In Vitro* Differentiation of TEs with *Z. elegans*

A number of parameters are important for establishing the *Z. elegans* differentiation system in a new lab. One of the most important aspects of making the *Z. elegans* tissue culture system work is the growth of the plants. Plants are grown in chambers with 16h of light, 8h of darkness at 27°C. After repair of the existing growth chambers we started growing our plants as close to the fluorescent and incandescent bulbs as possible but still maintaining good temperature control. Maximizing light intensity helps the plants to grow quickly. Primary leaves should be >4 cm within 12 days.



Other critical parameters for successfully getting TE differentiation *in vitro* with *Z. elegans* is the leaf sterilization and maceration techniques. We use low levels of bleach (0.05%) for 10 minutes. After sterilization, the epidermal surfaces of the leaves should still look glossy and not dark green. These low levels were used so as not to damage or kill leaf cells. The maceration process is necessarily damaging, but it is important to limit this damage and cell death. After maceration and washing > 50% of the cells must be viable for efficient differentiation to occur.

Optimization of TE Transdifferentiation of *Z. elegans* Mesophyll Cells

We have standardized the growth of *Z. elegans* plants to new soil mixes, fertilizers and growth chambers. Leaf sterilization and cell isolation were done successfully once good plant growth conditions were established. We are now getting normal rates of differentiation and normal timing for the onset of differentiation (Table 1).

Table 1

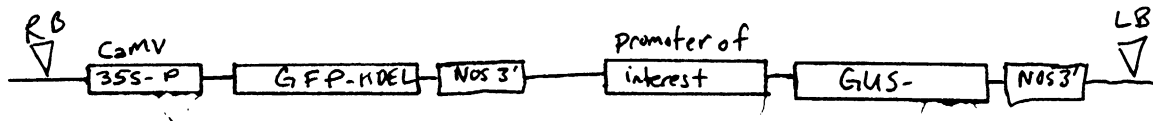
Experiment #	% of Tracheary Elements at 72H
1	55
2	50
3	45
4	62
5	55

Expression Vector Construction:

In order to determine gene regulatory elements, the subcellular locations of and to test for the functions of novel proteins by overexpression or antisense strategies we need to design and construct at least three separate expression vectors. To analyze for gene regulatory elements, the 5' and 3' sequences that flank the coding region of the gene are placed next to a reporter gene for example, β -glucuronidase (GUS) (Figure 1). In addition on this vector a control gene e.g., green fluorescent protein (GFP), is required to standardize the results from transformation efficiency. For subcellular localization studies a protein fusion vector to green fluorescent protein (Figure 2) is needed. This vector must contain a promoter that drives expression in differentiating TE's and cloning sites to fuse the protein of interest to either the amino terminus or the carboxyl terminus of GFP. A third vector is one where the gene of interest is placed downstream either in the sense or antisense direction of a promoter that is active in differentiating TE's (Figure 3). This vector also must have an control marker gene to identify which cells in the experiment were transformed.

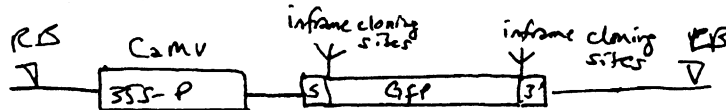


Figure 1: Vector to Identify Gene Regulatory Sequences



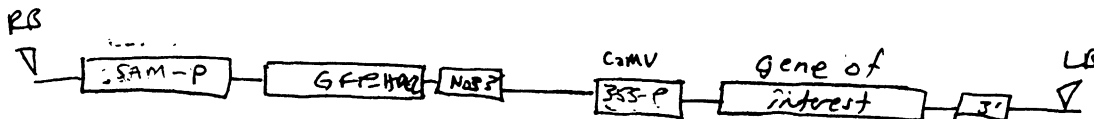
To identify gene regulatory sequences the vector we are constructing contains the SAM promoter driving the expression of GFP-HDEL (see below for a description) and the promoter of interest driving the expression of β -glucuronidase gene. The backbone vector is pBI 101 in which the NOS-NPT II gene has been deleted.

Figure 2: Vector for Subcellular Localization of Novel Proteins in Differentiating TEs



We are in the process of the constructing expression vectors for subcellular localization. For the subcellular localization experiments we propose to use the codon modified synthetic GFP, which enhances expression in humans and plant cells (Hass, Park, Seed 1996 Curr. Biol. 6: 315-324). This GFP will be driven by the CaMV 35S promoter and in frame cloning sites at both the 5' and 3' ends will be engineered into this vector. The backbone vector for these constructions will be pBIN 19 that have the NOS promoter and NPT II gene deleted from it.

Figure 3 Vector for Sense and Antisense Expression



For overexpression and antisense strategies to work a strong promoter needs to drive the expression of the gene of interest. We will use the CaMV 35S promoter, since I already know that this works well in differentiating TEs from *Z. elegans*. This vector also needs an internal reporter to mark cells that are transformed. The internal control for in our overexpression vector is a version of GFP with an HDEL sequence for localization to the endoplasmic reticulum (ER). This ER resident version of GFP is being used, because it removes the protein from the cytoplasm and minimizes any adverse or toxic effects that could occur due to its presence in the cytoplasm. For expression of this GFP we are testing whether the S-adenosyl methionine (SAM) synthase promoter is expressed in differentiating TEs from *Z. elegans*. The SAM promoter is highly expressed in the xylem tissues of *Arabidopsis thaliana*.

Testing of the Constructs in Transient Assays:

We are in the process of standardizing our transient assays so that we can test the SAM promoter and different versions of GFP. This work is just getting under way.



**EXTERNALLY FUNDED RESEARCH
in 1997-1998
SUPPORTING F011**

**John Cairney
Gary Peter
Luis Destefano
Chris Ricker**

March 26-27, 1998

Gene Expression in Trees: Sequences Affecting Stability/Translatability of mRNA

Luis Destéfano-Beltrán and John Cairney

Successful Genetic Engineering of Trees requires that a transferred gene continues to be expressed to the right degree and in the right location despite weather variations over the growing season. Environmental variations, such as drought or heat, 'close down' subsets of genes, favoring the expression of defense and repair genes most needed under those conditions. A gene, which conferred pest resistance would be useless if it functioned at 20% of its expected level whenever the tree was short of water.

Control of gene expression has to date focused on transcription, the synthesis of mRNA. Thus promoters which are inducible under desired conditions may be used to control transferred genes. However, the synthesis of protein encoded by an mRNA is under numerous controls and the quantity of mRNA may not correspond to the quantity of protein produced (Fig.1).

We have identified a sequence in a drought-inducible gene which we believe may permit mRNAs possessing this sequence to be translated optimally under conditions where most mRNAs are quiescent. We believe that this sequence can confer this property of selective translation upon adjacent foreign sequences i.e. genes of interest to tree breeders.

To demonstrate this principle we have cloned the 3' end of this gene next to the GUS reporter gene (Fig.2) and will transfer these constructs into plant cell lines. As seen in the figure, the effect of removing key sequences will be examined. These cell lines will be exposed to different levels of osmotic stress and different hormones and the modified transcript and its' translatability will be assayed.

In similar fashion, constructs containing different lengths of two different promoter sequence have been constructed and will be transformed into plants. We are interested in determining whether the stability/translatability sequence is influenced by its native promoter, compared to a constitutive promoter. Coding regions of the Proteinase Inhibitor gene have been cloned into an expression vector to determine what protection is afforded to transgenic plants by elevated amounts of the protein.

The extensive and meticulous work described above will allow us, in future months, to determine features important in the accurate and timely expression of a drought inducible gene, and by application of this knowledge allow us to design vectors which will optimize expression of genes of choice.





Factors Affecting Gene Expression

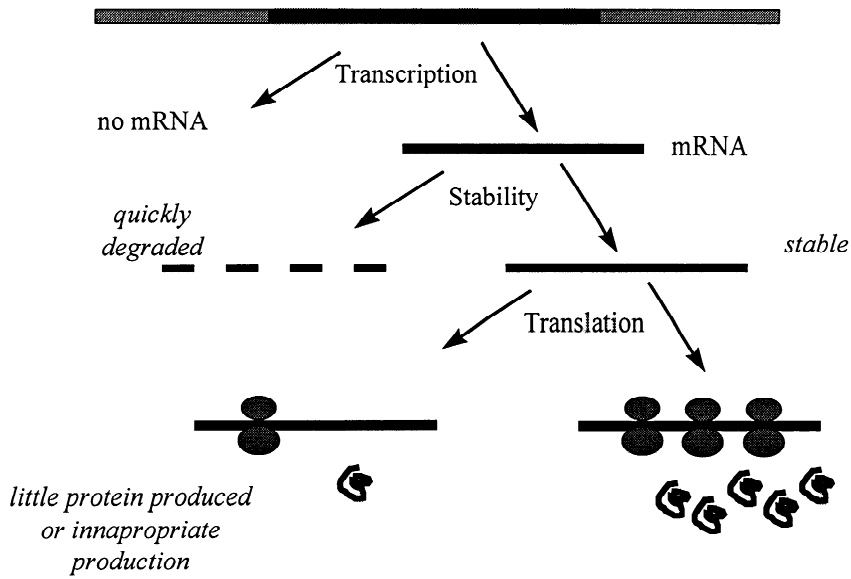


Figure 1



Construction of 35S-GUS-3'UTR 's

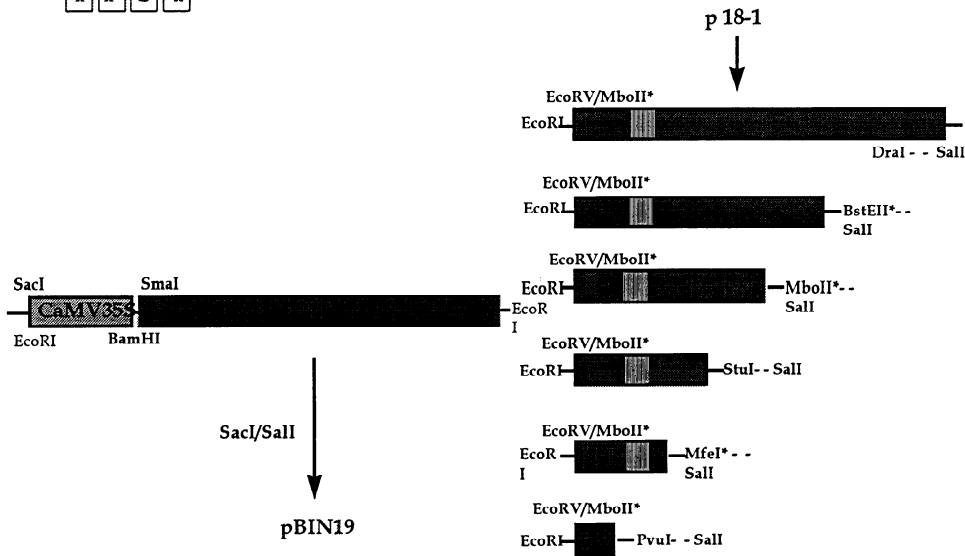


Figure 2



Gene Regulation in Woody Plants:

Identification of A Plant Enzyme Which Activates A Regulatory Peptide

Luis Destefano¹, Armando Casas-Mollano², Cody Cain⁴, Dana Freeman¹, Jerry Pullman¹, Sheldon May³, Charlie Oldham³, Allison Moore³, John Cairney¹

¹Institute of Paper Science and Technology, Forest Biology Group, 500 10th Street NW
Atlanta GA 30318. ²Visiting UNESCO Fellow

³Department of Chemistry and Biochemistry, ⁴Department of Biology, Georgia Institute of Technology, Atlanta

The continuation of work initiated as an A190 project with Georgia Tech has resulted in the isolation of several putative genomic clones, from an Arabidopsis library, for a plant counterpart of a regulatory enzyme which in mammals systems regulates the activity of many neuropeptides. Such work will enhance our understanding of plant regulatory systems and permit more specific manipulation of enzymatic activities in future work aimed at tree and general plant improvement. This project seeks in the short term to isolate and characterize such a gene from Arabidopsis, the model plant par excellence, and subsequently from loblolly pine.

The mammal PAM-PGL enzyme system

In mammals, many regulatory peptides require a modification -an amidation- at their carboxy terminal in order to become physiologically active. **PAM** (Peptidylglycine a-amidating Monooxygenase) and **PGL** (Peptidoaminoglycolate Lyase) work in close collaboration to modify and activate certain enzymes. Such mammalian enzymes target include calcitonin, growth hormone releasing factor, LH-RH (luteinizing hormone releasing hormone), vasopressin, gastrin, a-MSH (a-melanotropin) and others. The production of C-terminally amidated peptides can be better described as a two-step process in which a non-amidated peptide produced in the first step, is thereafter amidated at its carboxy end.

The monooxygenase PAM forms the α -hydroxyglycine derivative of the target peptide and the PGL catalyzes the dealkylation step to form the amide and glyoxylate products (Fig. 1)

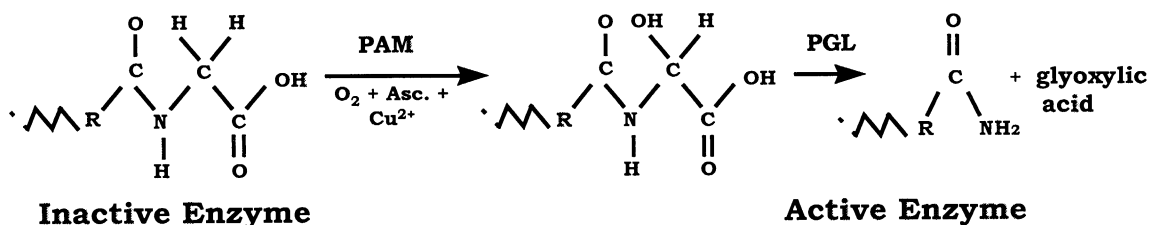


Figure 1. Mechanism of the PAM-PGL system.

In general the animal PAM and PGL enzymes are translated as a single peptide which is immediately self-cleaved into two active peptides. Several PAM-PGL cDNA and genomic clones have been isolated from several mammalian sources and their gene regulation has been specially investigated for the rat and human forms.

This project is jointly undertaken with GIT and provides an opportunity to determine novel forms of regulation, to establish an active collaboration with GIT, and to obtain extramural funding.

Early cell culture work showed that a highly specific inhibitor of PAM-PGL arrests growth of pine somatic embryos in a concentration dependent manner. More recently we have pursued this work following molecular approaches and conducted a number of Southern blot analysis to establish the presence of a PAM-PGL counterpart in plants. We did generate a 500 bp PAM fragment from a recombinant baculovirus clone from Dr. Mays lab and used it to probe genomic DNA from Loblolly Pine and Arabidopsis; although the loblolly pine gave a smeared signal the Arabidopsis gave somewhat more pronounced bands at low stringency.

This preliminary result supported the idea that a similar gene might indeed be present in plants and next we decided to screen a library. Since we had no idea as to when and where the gene might be expressed cDNA libraries were ruled out as a source for the clone. Our attention then focused on Arabidopsis because,

- Arabidopsis has a small genome with little repetitive DNA; genes are relatively easy to find
- Genomic libraries are available free of charge from the Arabidopsis Stock Center
- Genomic clones obtained from Arabidopsis will be a useful probe for isolating other plant copies e.g. from Loblolly Pine

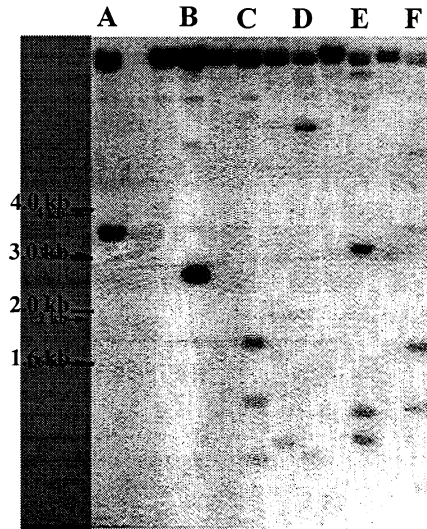
We have now obtained a full-length bovine cDNA clone from Dr. Richard Means, Johns Hopkins University. We have screened 66 000 clones (equivalent to just over three genomes) from an Arabidopsis genomic library. Our initial screening yielded about twenty faint signals and the corresponding plugs were isolated for secondary re-screening. A second round of purification focused on six clones, the remaining clones will be checked in future. DNA from these lambda clones was isolated, and digested, in separate experiments, with EcoRI, HindIII, or XbaI, releasing fragments ranging in size from 6kb to a few hundred base pairs. Gels were blotted onto nylon membranes and Southern blots were performed using the bovine PAM-PGL clone as a probe. Hybridization was performed under conditions similar to those used in plaque hybridization: hybridization at 50° C and four washes with 2X SSC, 0.1% SDS. All six clones showed one to several positive signals, confirming that the probe was detecting a specific sequence (Fig.2).

Figure 2. Southern Blotting of Enzyme Digested Lambda Clones Identified Hybridizing Fragments

Lambda DNA from several clones was digested with EcoRI (A) or with XbaI (B-F), separated on a 1% agarose gel and blotted onto N+ membrane.

Membrane was probed with the full length bovine PAM/PGL cDNA, washed 4 times with 2XSSC, 0.1% SDS and exposed to a film for 30 min at room temperature.

DNA from the positive clones has been shot-gun cloned into pBluescript. Several clones have the same size as the positive signals, they will be sequenced.



UPDATE: The 2.5kb fragment of Clone B shows a very strong signal with the probe, the intensity of the signal and the size of the fragment encouraged us to examine this clone first. Gels were run stained with Ethidium Bromide and the bands were excised and cloned into pUC19. Each clone was sequenced. The clone of the 2.5 kb fragment shows great similarity (46% nucleotide identity) over the length of the PAM-PGL sequence. This is a very high nucleotide similarity between an animal and a plant gene. Amino acid alignment gives lower similarity however this is because we are comparing a genomic clone (Arabidopsis) with a cDNA clone (Bovine PAM-PGL). We do not know where the introns are in our genomic clone and as reading frames can be shifted whenever we encounter an intron we cannot predict the sequence of the encoded protein. The only genomic clone available of PAM-PGL is from rat. This is very long (160kb) and has 27 introns. These introns permit multiple forms of PAM-PGL to be generated by alternative splicing. It would not be surprising if the Arabidopsis clone, despite its' much smaller size, possesses many introns. The isolation of a cDNA clone from Arabidopsis allows assignment of exons and introns to the sequence and would thus permit amino acid alignment. Such a clone is now being sought. Since the specific inhibitors of PAM-PGL were effective with Pine liquid suspension culture we will determine whether they inhibit growth of Arabidopsis culture and whether a specific mRNA can be detected by Northern analysis. If these experiments prove encouraging, a cDNA library will be constructed from Arabidopsis suspension culture and screened. Similar experiments can be conducted with Pine systems, where we have number of cDNA libraries already made.

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: \$142,249 (\$121,249 to IPST, and \$21,000 to GIT)
 Period of Proposal: Fiscal Year 1998 (July 1, 1997 - June 30, 1998)
 Submitted: 12th July 1996
 Status: **Awarded \$ 109,249 to IPST, \$21,000 to GIT**

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: \$84,622 (IPST)
 Period of Proposal: Fiscal Year 1998 (July 1, 1997 - June 30, 1998)
 Submitted: 12th July 1996 Status: **Awarded \$ 73,622**

Title: Molecular Manipulation of Reproduction in Southeastern Tree Species of Commercial Importance
 Authors (Affiliation): Jeffrey F. 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: \$355,448 (\$180,119 to IPST and \$175,329 to UGA)
 Period of Proposal: Fiscal Year 1998 (July 1, 1997 - June 30, 1998)
 Submitted: July 15, 1996
 Status: **Awarded \$136,163 to IPST, \$160,620 to UGA**

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: \$42,644
 Period of Proposal: 1997
 Submitted: September 17, 1996, Status: **Awarded, \$42,644 to IPST**

Title: The Role of Peptide Processing in Plant Cell Growth and Development
 Authors (Affiliation): Sheldon W. May (GIT), Gerald Pullman (IPST), and John Cairney (IPST)
 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: 1996-1997
 Submitted: August 30, 1996
 Status: **Grant Awarded (\$20 K to IPST Forest Biology, \$20 K to GIT Dept. Chemistry & Biochemistry)**

Title: The Role of Calcium Dependant Protein Kinases in Xylem Tracheary Element Differentiation and Zygotic Embryos of Loblolly Pine
 Authors (Affiliation): Gary Peter (IPST), and Jung 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: 1996-1997
 Submitted: August 30, 1996

Status: **Grant Awarded (\$20 K to IPST Forest Biology, \$20 K to GIT Dept. of Biology)**
 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: Fiscal Year 1997 (July 1, 1996 - June 30, 1997)
 Submitted: February 1, 1996
 Status: **Awarded \$72,000 to IPST**

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: **Awarded \$98,779 to IPST**

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: **Awarded \$73,500 to IPST**

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: **Awarded \$68,000 to IPST**

Pending Proposals

Title: Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expression Studies.
 Authors (Affiliation): Nanfei Xu (IPST), G. Pullman (IPST), J. Cairney (IPST)
 Awarding Agency: Traditional Industry Program in Pulp and Paper (The Georgia Consortium for Technological Competitiveness in Pulp and Paper)
 Amount Requested: \$144,377
 Period of Proposal: Fiscal Year 1999 (July 1, 1998 - June 30, 1999)
 Submitted: 1st January, 1998 Status: in review

Title: Drought Protection in Forest Trees: Post-Transcriptional Regulation of Two Stress-Responsive Genes
 Authors (Affiliation): John Cairney (IPST), Gerald Pullman (IPST), Luis Destéfano-Beltrán
 Awarding Agency: Traditional Industry Program in Pulp and Paper (The Georgia Consortium for Technological Competitiveness in Pulp and Paper)
 Amount Requested: \$90,065 (IPST)

Period of Proposal: Fiscal Year 1999 (July 1, 1998 - June 30, 1999)
 Submitted: 1st January, 1998 Status: in review
 Title: Environmental Influences and Genetic Regulation of Microfibril Angle in Southern Pine
 Authors (Affiliation): Gary Peter (IPST) and Timothy Faust (UGA)
 Awarding Agency: Traditional Industry Program in Pulp and Paper (The Georgia Consortium for Technological Competitiveness in Pulp and Paper)
 Amount Requested: \$83,823 (\$70,323 to IPST and \$13,500 to UGA)
 Period of Proposal: Fiscal Year 1999 (July 1, 1998 - June 30, 1999)
 Submitted: 1st January, 1998
 Status: in review

 Title: Molecular Manipulation of Reproduction in Southeastern Tree Species of Commercial Importance
 Authors (Affiliation): Jeffrey F. Dean (UGA), Scott A. Merkle (UGA), Sarah Covert (UGA), Gerald Pullman (IPST), and John Cairney (IPST)
 Awarding Agency: Traditional Industry Program in Pulp and Paper (The Georgia Consortium for Technological Competitiveness in Pulp and Paper)
 Amount Requested: \$278,681 (\$164,251 to IPST and \$114,430 to UGA)
 Period of Proposal: Fiscal Year 1999 (July 1, 1998 - June 30, 1999)
 Submitted: 1st January, 1998
 Status: in review

 Title: Loblolly Pine Embryogenesis - A Study of Early Development Genes
 Authors (Affiliation): Nanfei Xu, J. Cairney, and Gerald Pullman
 Awarding Agency: USDA - National Research Initiative Competitive Grants Program
 Amount Requested: Years 1 \$70,001, Year 2: \$72636.
 Period of Proposal: Fiscal Years 1999, and 2000
 Submitted: December 15, 1997
 Status: In review

 Title: Drought Protection in Forest Trees: Post-Transcriptional Regulation of Stress-Responsive Genes.
 Authors (Affiliation): J. Cairney (IPST), L. Destefano (IPST), A. Altman (HUJ), O. Shoseyov (HUJ)
 Awarding Agency: BARD, USDA-ARS-OIRP (U.S. - Israel)
 Amount Requested: IPST Years 1-3: \$69.1K, 67.9 K, 71.4 K; HUJ Years 1-3: \$54.6 K, 55.8 K, 58.8 K.
 Period of Proposal: Fiscal Years 1998, 1999, and 2000
 Submitted: August 30, 1997
 Status: In review

 Title: Transformation of loblolly pie: *Agrobacterium tumefaciens* gene transfer and improved selection, evaluation, and plant regeneration using somatic embryogenesis.
 Authors (Affiliation): A. Wenck (NCSU), G. Pullman (IPST), and G. Peter (IPST)
 Awarding Agency: Preproposal - Agenda 2020 - Sustainable Forestry Program, U.S. DOE
 Amount Requested: Year 1 \$123,830 (\$59,830 to IPST, \$64,000 to NCSU), Year 2 \$118,820 (\$62,820 to IPST, \$56,000 to NCSU)
 Period of Proposal: Fiscal Years 1999 and 2000
 Submitted: Original submission August 26, 1997, Second submission 21 January, 1998
 Status: In review

Title: Fast Growing Trees Through Biotechnology: An Alternative High Value Crop.
 Authors (Affiliation): G. Peter, J. Cairney and G. Pullman
 Awarding Agency: Agenda 2020 Sustainable Forestry Program
 Amount Requested: Year 1 \$117,315, Year 2 \$118,906, Year 3 \$117,726
 Period of Proposal: Fiscal Years 1998 - 2000
 Submitted: Original submission August 26, 1997, Second submission 21 January, 1998
 Status: In review

Title: A Loblolly Pine Mutant for Simplified Lignin Removal
 Authors (Affiliation): J. MacKay, D. Dimmel, G. Pullman, and G. Peter
 Awarding Agency: Preproposal - Agenda 2020 - Environmental Group, U.S. DOE
 Amount Requested: Year 1 \$158,087 and Year 2 \$165,992
 Period of Proposal: Fiscal Years 1998 and 1999
 Submitted: July 1, 1997
 Status: In review

Title: Molecular analysis of male and female cottonwood trees.
 Authors (Affiliation): David E. McMillin (Clark Atlanta Univ.) and John Cairney (IPST)
 Awarding Agency: TAPPI
 Amount Requested: \$40,000 (\$20,000 IPST and \$20,000 CAU)
 Period of Proposal: October, 1997-November 1998
 Submitted: June 27, 1997
 Status: In review

Rejected Proposals

Title: Fast Growing Trees Through Biotechnology: An Alternative High Value Crop
 Authors (Affiliation): G. Peter, J. Cairney and G. Pullman
 Awarding Agency: Rural America Fund - Department of Agriculture
 Amount Requested: Year 1 \$168,658 and Year 2 \$178,590
 Period of Proposal: Fiscal Years 1997 - 1999
 Submitted: April 25, 1997
 Status: Not Funded

Title: Trees Containing Built-In Pulping Catalysts
 Authors (Affiliation): Gerald Pullman, Don Dimmel, John Cairney (all IPST)
 Awarding Agency: Preproposal - Agenda 2020 - Capital Effectiveness, U.S. Department of Energy.
 Amount Requested: \$137,335.65
 Period of Proposal: 1997 Fiscal Year
 Submitted: August 31, 1996
 Status: Not Funded

Title: Signaling Mechanism that Coordinates Lignin Biosynthesis - A General Approach to Regulated Decreases of Lignin Content in Trees.
 Authors (Affiliation): Gary Peter, Gerald Pullman, John Cairney (all IPST)
 Awarding Agency: Preproposal - Agenda 2020 - Capital Effectiveness, U.S. Department of Energy.
 Amount Requested: \$113,407
 Period of Proposal: 1997 Fiscal Year
 Submitted: September 9, 1996 Status: Not Funded

The Georgia Consortium for Technological Competitiveness in Pulp and Paper

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

2. Investigators:

Co-Principal Investigators:

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3. Executive Summary:

This proposal seeks continued funding of this project for FY99. This project was initiated November 8, 1996 with a grant of \$75,000 to IPST as State of Georgia Bond Funds (FY96). Due to special bond fund requirements spendable \$ did not become available to IPST until January 19, 1996. Work during the first six months of 1996 was highly successful in developing a method to compare differential gene expression in developing conifer embryos. Funding continued for FY97 (\$ 98,779) resulting in special techniques to accommodate the minute amounts of tissue present in developing loblolly pine embryos. As little as 20 embryos isolated shortly after fertilization or one late staged embryo produced repeatable patterns of co-migrating bands elucidating gene activity. Using the developed techniques a pattern of gene expression activity for many genes was presented throughout embryo development. Funding continued for FY98 (\$ 107,249). During FY98 many RNAs were identified which change in abundance at different stages of somatic and zygotic embryo development.

Many of the genes were isolated, sequenced, and entered into the genebank for identification. The GenBank database indicates that most of these are novel sequences, although a number of putative regulatory proteins have been identified which are expressed at different stages of embryo development. Further, a highly sensitive and reliable Dot-Array Southern protocol has been developed that can be used to examine the expression of several hundred genes simultaneously in a single embryo. This technique is now ready for use to improve somatic embryo quality.

A continued supply of low-cost, high quality raw materials is essential for the future success of the U.S. Forest Products Industry. The clonal propagation of high value trees through somatic embryogenesis has the potential to help meet future industry needs by increasing forest yields and improving raw material uniformity and quality. Somatic embryos of loblolly pine can be produced currently but quality is inferior when compared to natural seed embryos. In somatic embryogenesis the many changes that occur during development are difficult to observe visually. By contrast, the molecular events are dramatic and conspicuous. We have cloned 417 cDNAs that represent genes that are expressed at different stages of embryogenesis in loblolly pine. In this comprehensive program, the expression of these genes in the somatic embryos will be monitored and compared to the expression pattern in zygotic embryos at equivalent stages. Tissue culture treatments will be applied to somatic embryos at different stages, and the effects of these treatments on the expression of the 417 marker genes will be assessed. By analyzing this data, it will be discovered which factor(s) in the culture medium regulate (turn on or off) individual marker genes. This vital information can then be used to design a tissue culture method or methods that allow the expression of the marker genes in the somatic embryos to closely match that in the zygotic embryos on a stage to stage basis. When the gene expression in somatic embryos resemble zygotic embryos stage by stage, the quality of the somatic embryo should be similar to the target zygotic embryo. This approach is possible at IPST because we now have the largest collection of embryogenesis-related cDNAs, and we have developed highly sensitive and reproducible methods to reliably detect the expression of these genes in minute amount of plant tissues.

There may be some genes that cannot be turned on or off by manipulating tissue culture conditions. At least some of these genes are expected to play an important role in controlling embryo quality. These genes will be studied in detail. The full length cDNA and the genomic sequences of these genes will be isolated and sequenced. The possible function of the gene products and the developmental regulation of these genes will be explored through transgenic studies. This work will generate fundamental knowledge in plant embryogenesis and yield important clues for development of commercial protocols for the clonal propagation of high-value trees in Georgian tree plantations and forests.

Key Words: Somatic Embryogenesis, Zygotic Embryogenesis, High-density cDNA array, Gene Expression, Gene Regulation, Control Gene Expression, Embryo Quality.

The Georgia Consortium for Technological Competitiveness in Pulp and Paper

1.- **Title:** **Drought Protection in Forest Trees: Post-Transcriptional Regulation of Two Stress-Responsive Genes**

Institution: **Institute of Paper Science and Technology (IPST)**

2.- **Investigators:**

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3.- Executive Summary

The establishment of **plant genetic engineering** at the forefront of plant biotechnology has resulted in the generation of many plant species with novel genes. These achievements have been recently paralleled by the development of regeneration and transformation protocols for a number of tree species including several forest trees (Tzfira *et al.* 1997, *Physiol. Plant.* **99**: 554). Consequently, in the near future new forest tree genotypes with engineered characters will become a marketplace reality. This proposal describes work directed towards ensuring that the valuable, desirable genes that are transferred into trees will be expressed correctly when trees are growing under stress.

While successful gene transfer is facile for many species, the consistent expression of a transferred gene is not a foregone conclusion. Some of the “rules” for obtaining good levels of expression are lately becoming clear. In addition to a suitable promoter for gene transcription in the desired tissue or growth conditions, specific untranslated sequences at either end of the mRNA molecule have been recognized which determine stability, translation or localization of mRNA, and thus optimize protein production (Koziel *et al.* 1996, *Plant Mol. Biol.* **32**: 393). Control of gene expression, operating after RNA is produced, is known as **post-transcriptional** regulation. This swift and flexible form of gene regulation is relatively understudied, compared to, say **transcription**, but it has a central role in plant growth and development especially for generating a **fast response** to environmental and intracellular signals. Accordingly, Post-transcriptional processes have an important application in biotechnology for the development of plant expression vectors which ensure correct expression of transferred gene sequences.

The plant losses associated with **drought susceptibility of young loblolly pine seedlings** are significant problems for forest establishment and growth in the state of Georgia. Further, several companies report difficulties in taking a high-value hardwood clone which grows well in Site A, and establishing a productive stand at Site B in another part of the State.

This proposal focuses on the study of **two drought-responsive genes** which are regulated by distinctive post-transcriptional mechanisms. The first gene is a member of a multigene family of proteinase inhibitors (PI's) and is up-regulated by drought-stress from *Atriplex canescens*. This gene possesses an AU-rich sequence in its 3'-UTR. Similar AU motifs have been implicated in the regulation of the stability and translatability of other RNA messages (Abler and Green 1996, *Plant Mol. Biol.* **32**: 63). We speculate that this AU-rich 3'-UTR placed downstream of the coding sequence of a reporter gene may regulate its stability and translatability and that when incorporated into a vector, this sequence would enhance the amount of protein produced from the gene of choice.

The second is represented by LP6, a drought-stress regulated gene from loblolly pine (*Pinus taeda*). Its unusual long leader includes eight small **upstream open reading frames**, uORF's, and a number of potential stem-loops (Chang *et al.* 1996, *Plant Mol. Biol.* **31**: 693). In mammalian and more recently in plant systems such features have been implicated in the translational control of their downstream messages (Gallie 1996, *Plant Mol. Biol.* **32**: 145). We hypothesize that any coding sequence placed downstream of this long and unusual 5'-UTR may also be regulated in a post-transcriptional manner.

Understanding how to regulate genes during drought will be an important part of this project. Findings and genetic material isolated are expected to be useful for transformation in both hardwoods and softwoods. This project represents **novel approaches to the genetic improvement** of southern softwood and hardwood species and hold enormous potential for improving forest health and productivity.

Key Words: Environmental Stress, Drought, Biotechnology, Tolerance, Stress-related Genes, Transgenic Plants, Plant Transformation, Post-transcription regulation, vector design

TRADITIONAL INDUSTRY PROGRAM IN PULP AND PAPER (TIP³)
FY1999

ENVIRONMENTAL INFLUENCES AND GENETIC REGULATION OF
MICROFIBRIL ANGLE IN SOUTHERN PINE

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The University of Georgia

I. EXECUTIVE SUMMARY:

Microfibril angle (MFA) is considered to be one of the most important fiber characteristics controlling paper properties. The proposed research will develop techniques to rapidly and simply measure MFA. These methods will be used to expand our knowledge of the effect of silvicultural treatment and genetic control of MFA in southern pine. The data obtained on MFA and fiber length in this work will be analyzed together with the existing data on wood density, pulp yields and paper properties previously obtained by T. Faust from wood grown with specific silvicultural treatments. When both sets of data are integrated together, we should get a fundamental understanding of how silvicultural treatment can affect wood quality and what individual traits are coordinately changed by maximizing growth rate in both slash and loblolly pine. We also propose to collaborate with breeders to begin screening populations of loblolly pine for trees that have lowered MFAs in juvenile tracheids and to identify linked DNA markers that segregate with this important trait. Finally, we propose to initiate very fundamental studies to begin elucidating the molecular mechanisms that regulate microfibril angle.

Southern pine trees harvested after only 12-15 years are mostly juvenile wood and contain shorter, thinner walled fibers with high microfibril angles when compared with mature fibers. For southern pine tracheids or fibers, strength is the most important attribute. Strength is determined by a combination of fiber length, secondary cell wall thickness and MFA. Southern pine juvenile fibers are long and have relatively thick secondary cell walls but high MFAs. MFA is the angle that cellulose microfibrils are deposited relative to the long axis of fiber cells. Fibers that have a low angle are stronger and exhibit less shrinkage than fibers that have higher angles; lowering the MFA by 10° represents a 25% increase in fiber strength. Trees with lower and more uniform MFA are highly desired because both wood and paper products made from them have improved dimensional stability due to the enhanced fiber strength and decreased shrinkage. Lowering the MFA in juvenile wood should increase the value of wood and paper products made from these younger plantation grown trees. The forest products industry adds 17.3 billion dollars to Georgia's economy. The potential to increase that revenue with value-added products is many times that amount. The improvement or manipulation of fiber quality is key to facilitating the development and competitive manufacturing of value-added products.

Planting trees that have superior growth rates in Georgia as well as improved wood and fiber properties is critical for the long term success and sustainability of the Georgia pulp and paper industry. Growers and breeders of trees are making important and far reaching decisions about what genotypes to improve and grow on future plantations. They are also trying to determine if the silvicultural treatments even for existing stands can have dramatic effects on growth rate as well as on wood and fiber quality. Understanding how growth conditions and genetic background effect wood and fiber quality will help companies maximize their existing and future forest resources. The information obtained from the proposed research will help pulp and paper companies to make informed decisions about what genotypes to use, how to breed for better genotypes, and how to use silvicultural treatments that maximize growth as well as improve fiber length and microfibril angle. This research will help companies to grow higher value trees faster, both now and in the future. These decisions are vital for Georgia growers and forest products companies to stay competitive in the global market and to improve the value of their existing products.

KEY WORDS: cellulose microfibril, environmental, fiber quality, genetic, loblolly pine, microfibril angle, microscopy, silviculture, slash pine



**TRADITIONAL INDUSTRY PROGRAM
IN
PULP AND PAPER (TIP3)**

**FIBER SUPPLY DIVISION
FY 99 PROJECT APPLICATION**

1. TITLE: *Molecular Manipulation of Reproduction in Southeastern Tree Species of Commercial Importance*

2. INVESTIGATORS:

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3. EXECUTIVE SUMMARY

Progress in genetic improvement of forest trees has been significantly slower than that in agronomic species due to the large size of trees and their typical lag to sexual maturity. Not only have these problems limited the pace of improvement for trees of commercial importance, but they have limited genetic improvement programs to a mere handful of species. Genetic engineering can be used to introduce unique genes into elite tree backgrounds, thereby speeding tree improvement. However, many genes that hold great potential for addressing immediate problems (e.g. *Bt* toxin and herbicide-resistance genes) could create new problems if allowed to escape into wild tree populations. Thus, sexual reproduction creates at least two severe bottlenecks to progress in forest tree improvement. Fortunately, research on the control of floral tissue development in herbaceous plants has suggested new possibilities for eliminating both of these bottlenecks. This research program seeks to develop techniques for controlling the development of reproductive tissues in southeastern forest tree species, both for the production of trees that flower at an early age, as well as for the production of sterile tree lines.

Precocious Flowering: *Arabidopsis thaliana* LEAFY and APETALA genes under the control of constitutive (CaMV 35S) and inducible (Cu²⁺) promoters were used to transform embryogenic cell lines of yellow-poplar. Transgenic trees that expressed these genes in a constitutive manner were regenerated and transferred to the greenhouse. No precocious flowering has been noted to date. Work for FY99 is anticipated to focus on: 1) regeneration and testing of transgenic trees harboring inducible versions of these genes; 2) cloning and characterization of full-length homologs of the floral homeotic genes from each of the target species; 3) testing of the tissue-specific expression patterns for each of the endogenous homologs; and 4) transformation of target species with these homologs in order to develop early flowering trees.

Sterility: Promoters that regulate expression of the LEAFY and APETALA genes in *Arabidopsis thaliana* were fused to the β -glucuronidase (GUS) reporter gene, as well as the DTA cytotoxin gene. However, transient expression assays with the GUS constructs showed that the APETALA promoter was active in embryogenic cells of both yellow-poplar and sweetgum, and thus will not be useful for work with these species. Similarly, homologs of floral homeotic genes were found to be expressed in embryogenic cultures of loblolly pine. Work for FY99 is anticipated to focus on: 1) identification and characterization of endogenous promoters that control expression of genes in reproductive tissues other than embryos; and 2) further study of the expression of homeotic genes in the development of embryos and embryogenic cell cultures of loblolly pine.

This collaborative effort between researchers at the University of Georgia and the Institute for Paper Science and Technology is designed to assure that techniques for inducing precocious flowering and sterility will be developed as rapidly as possible for the premier commercial tree species of the southeastern U.S. (loblolly pine), as well as for hardwood species of commercial interest. Techniques for inducing precocious flowering and sterility hold enormous potential for the forest products industry, and in the case of sterility, represent an enabling technology for future efforts to release genetically engineered trees for commercial production. Controlled precocious flowering would allow us to rapidly fix new traits introduced by gene transfer into a particular genetic background, and to move them into new backgrounds through greatly speeded breeding programs. However, we will be prevented from releasing genetically engineered trees into 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

PROPOSAL TYPE

- Standard Research Proposal
- Conference
- AREA Award
- Postdoctoral
- New Investigator
- Strengthening:**
- Career Enhancement
- Equipment
- Seed Grant
- Standard Strengthening

Principal Investigator(s):

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PI #2 Dr. John Cairney Institution Institute of Paper Science and Technology
PI #3 Dr. Gerald Pullman Institution Institute of Paper Science and Technology
PI #4 _____ Institution _____

Project Title: Loblolly pine embryogenesis--a study of early developmental genes

Key Words: Gymnosperm, gene expression, somatic embryo quality, gene regulation

(Approximately 250 words)

PROJECT SUMMARY

Defining the early events in embryogenesis is key to understanding embryo development. Gene expression early in embryogenesis has proven difficult to investigate in gymnosperms and angiosperms because embryos are small and buried in maternal tissue and molecular techniques have not been sufficiently sensitive. By modifying differential display we have been able to probe gene expression from the earliest stages of pine zygotic embryogenesis and have isolated over 400 differentially expressed cDNA clones. Somatic embryogenesis is a tool to study developmental processes and, especially in conifers, a very promising technique for the multiplication of superior genotypes. Many important conifers, however, are recalcitrant in tissue culture. Our panel of cDNAs will be used to probe gene expression in zygotic and somatic embryos of Loblolly Pine revealing induction of specific enzymes. Gene expression in genotypes with 'high' and 'low' embryogenic potential may now be determined and the effect of hormones or other culture conditions, assayed. This information can be used to construct testable hypotheses and from these, improve culture conditions and somatic embryo quality. Since a number of our pine cDNAs bear strong similarity to sequences from other plants we will be able to use these early expressed genes to probe gene expression in angiosperms and to reveal molecular similarities in angiosperm and gymnosperm embryogenesis. We intend to demonstrate the temporal and spatial expression of pine early expressed genes by RT-PCR and immunolocalization of proteins, in zygotic and somatic embryos and to identify regulatory sequences by promoter analysis of Pine and Arabidopsis promoters.

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.

Levi Barclay (M. Sc.) * Second year student.

Title: Using Microscopy and an Immunocytochemical Assay of Bromodeoxyuridine to Evaluate Liquid Culture Cell Division Rates

Advisor: Gary Peter

Summary: A method for using microscopy to evaluate liquid culture cell division rates by analysis of BrdU incorporation into DNA will be developed. This method should enable us to determine how quickly cells are dividing in liquid culture embryos as well as the relative cell division rate of the non-embryonic material in the culture. An immunocytochemical assay to determine the concentration of BrdU in a DNA sample will also be developed. This assay should allow us to develop a correlation between DNA production and cellular division rates. These methods will be applied to different genotypes and liquid cultures grown at different temperatures to measure their cellular division rates.

Douglas Benton (M. Sc.) First year student.

Title: Determination of the Microfibril Angle in the S2 Layer of Commercially Important Trees Using Differential Interference Contrast Microscopy

Advisor: Gary Peter

Summary: The microfibril angle (MFA) of the S2 layer in plant cell walls has been shown in many studies to be an important parameter in determining both fiber and paper's strength, stiffness, and shrinkage characteristics. Unfortunately, current methods used for measuring the MFAs from core samples are often time consuming, arduous and require expensive equipment. The purpose of this project is to develop Differential Interference Contrast Microscopy (DIC) as a reliable and less time consuming method to accurately determine the MFA.

Validation of DIC will be done by measuring the MFAs in standard fiber samples using DIC, then comparing these results to those obtained from two proven techniques: polarizing light microscopy and confocal laser scanning microscopy. If DIC proves to be a valid technique for measuring the MFA, it will then be used to investigate the range MFAs in commercially important angiosperm and gymnosperm genotypes obtained from short rotation plantations under various growing conditions. Data obtained from this study will help to address quality issues pertaining to short rotation fiber supply.

Karen Crews (M. Sc.) Second year student.

Title: A survey of Anthraquinones in Commercial Trees and their Potential Release from Chips during Pulping

Advisor: Jerry Pullman and Don Dimmel

Summary: Anthraquinones (AQs) can be used at extremely small amounts to improve pulping productivity and environmental factors. AQ increases pulping rates and product yields, removing greater amounts of lignin during pulping. Presently an AQ extract has been found in teakwood as well as several other angiosperms. One goal of this project is to expand our knowledge of AQ content in other tree species. Knowing if commercial tree species contain AQ may give us the knowledge that the pathway for AQ production is present. This will allow us to perhaps "turn up" the particular gene for AQ production in major pulping tree species. Using the trees that contain AQs to facilitate pulping of loblolly pine or another major pulping softwood would be the next step. A tree containing AQ components could be added to a major softwood pulp source, such as loblolly pine. These could be pulped together to promote delignification of the latter. Alternatively, liquors from a hardwood cook that contained AQs could be added to a softwood cook. Such experiments will be done with teak and pine. Other comparisons that will be made will be between known woods that are easily pulped and woods that are hard to pulp. If AQ is determined to have an effect on the ability to pulp commercial trees using the above experiments, altering the gene that produces AQ to generate more would allow for faster and more productive cooks for both AQ containing trees as well as non-AQ containing trees. The discovery of new pulping techniques, as well as a broader knowledge of the content of tree extracts, are possible benefits from this research.

John Ceranski (M. Sc.) Second year student.

Title: Fluorescent Microscopy: A Tool for Determining the Spatial Distribution of Secondary Wall Components Remaining during Kraft Pulping of Loblolly Pine

Advisor: Earl Malcolm and Gary Peter

Summary: The goal of this study is to determine the sequence of lignin and hemicelluloses removal from specific cell types, regions of cells and secondary cell wall layers during Kraft pulping of *Pinus taeda*. The removal and spatial organization of the remaining components at various times during pulping will be characterized with fluorescent probes to specific cell wall components and fluorescent microscopy in thin sections of pulped pine.

Douglas Mancosky (M.Sc.) First Year Student

Title: Temporal and Spatial Analysis of Gene Expression During Pine Zygotic Embryogenesis

Advisor: John Cairney

Summary: The proposed study will select a known clone and study gene expression at the mRNA and protein level for different stages of development. This will be accomplished using Northern blotting, RT-PCR and antibody probing. We will study gene expression *in situ*, determining where within the embryo genes are expressed, as well as at what stages of development expression occurs. Three clones, previously

isolated and sequenced and shown to bear homology with well characterized regulatory genes from other organisms have been selected for this study. These clones will serve as markers for embryo development and their detailed study will reveal many of the regulatory features which determine the timing and localization of gene expression during embryo development.

Michael Sullivan (M. Sc.) * Second year student.

Title: Assessing Gene Expression Changes During Culture Cycling

Advisor: John Cairney

Summary: Changes in the settled cell volume of liquid suspension cultures have been observed for many Loblolly Pine genotypes. The volume of settled cells follows a pattern, diminishing to a nadir then rising once more to peak at close to (but often below) the previous high. This cycling may be part of a trend of deterioration; maximum cell volumes never matching a previous high, minimum volumes being lower than the previous low. We wish to learn more about this phenomenon, both to follow the cultures through phases of a cycle and to determine similarities and differences between different peaks, different troughs and between peaks and troughs. Differential Display will be employed as a means of following biochemical changes, as manifest in changes in gene expression. Patterns of gene expression will be determined and potential 'marker' bands will be cloned and analysed.

Vincent Ciavatta (Ph. D.) * Ph.D. candidate.

Title: Analysis of Gene Expression During Development of Somatic and Zygotic Embryos.

Advisor: John Cairney

Summary: As a tool to follow Embryogenesis in vitro and in vivo, gene expression during equivalent stages of development will be compared. Differential Display will be used and patterns diagnostic of a particular stage of development for a particular genotype will be generated for somatic embryo. Bands which appear characteristic of early or late development or of a particular stage will be cloned and characterized. Equipped with the technical expertise and physiological insight which this will give us, similar experiments will be conducted with zygotic embryos. Comparing and contrasting gene expression in somatic and zygotic embryos will allow us to evaluate their state of health and permit informed modifications to media which should improve quality and quantity of embryos generated in vitro.

Stephen Van Winkle (Ph. D.)* Ph.D. candidate.

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.

IPST FOREST BIOLOGY TEAM PUBLICATIONS - 1996-1998
(Issued, in press, or submitted, * = work done at IPST)

1998

- *Leyva, A., Dimmel, D.R., and Pullman, G.S. 1998. Teak Extract as a Catalyst for the Pulping of Loblolly Pine. Tappi Journal. (In press.)
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- Whetten, R. W., MacKay, J. J. and ., Sederoff, R. R. 1998 Recent advances in lignin biosynthesis. Ann. Rev. Plant Physiol. & Plant Mol. Biol. (in press)

1997

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- *Cairney, J., G. S. Pullman, N. Xu, V. Ciavatta, and B. Johns. 1997. Differential Display: A tool to follow natural and somatic embryo development in loblolly pine. TAPPI Biological Sciences Symposium, October 19-23, 1997, San Francisco, CA. Pages 85-91. (ISBN 089852-695-7) TAPPI Press.
- *Cairney, J., Xu, N., Ciavatta, V., Johns, B., Pullman, J. (1997) Differential display as a tool to monitor embryo development in loblolly pine. Oral presentation to Joint Meeting of the IUFRO Working Parties, Somatic Cell Genetics and Molecular Genetics of Trees, Quebec City, Quebec, Canada.
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- MacKay, J. J., D. M. O'Malley, T. Presnell, F. L. Booker, M. M. Campbell, R. W. Whetten & R. R. Sederoff (1997) Inheritance, gene expression and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase. *P.N.A.S. USA* 94: 8255-8260
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