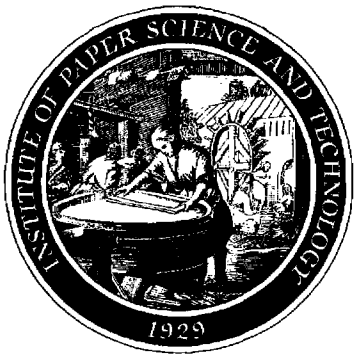

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Loblolly Pine Somatic Embryogenesis: Development of a Maturation Medium and Resulting Embryo Quality

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Loblolly Pine Somatic Embryogenesis: Development of a Maturation Medium and Resulting Embryo Quality

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Abstract

Clonal production of loblolly pine (*Pinus taeda* L.) through somatic embryogenesis has great potential to meet the increasing industrial demands for high-quality uniform raw materials. A major barrier to the commercialization of this technology is the low quality of resulting embryos. Twenty-five newly initiated loblolly pine genotypes were followed through the process of liquid culture establishment, embryo maturation, germination, and retrieval from cryogenic storage. A maturation medium, capable of promoting development of loblolly pine somatic embryos that can germinate, is presented that combines ½ P6 modified salts, 2% maltose, 13% polyethylene glycol 8000, 5 mg/L abscisic acid, and 2.5 g/L Gelrite. A procedure for converting and acclimating germinants to growth in soil and greenhouse conditions is presented. A set of somatic seedlings, produced from the maturation medium, showed

100% survival when planted in a field setting. Somatic seedlings showed normal yearly growth when compared to standard seedlings from natural seed. Quality of resulting embryos is examined and compared to zygotic embryos using morphology, dry weight, germination performance, and gene expression. All observations support the conclusion that somatic embryos grow approximately halfway through the normal sequence of development and then prematurely discontinue growth.

Key words: somatic embryogenesis, loblolly pine, Pinus taeda, embryo maturation, conifer

Introduction

A continued supply of low-cost, high-quality fiber is essential for the future success of the U.S. forest products industry. With the worldwide demand for paper expected to increase nearly 50% by the year 2010, efforts are increasing to boost forest productivity by propagating superior trees. Clonal propagation of high-value forest trees through somatic embryogenesis (SE) has the potential to rapidly capture the benefits of breeding or genetic engineering programs and to improve raw material uniformity and quality. Loblolly pine is dominant on 11.7 million ha. and comprises over half of the standing pine volume in southern U.S. forests due to its fast growth, broad natural range, response to cultural practices, resistance to disease and ice damage, and genetic variability for breeding (Westvaco 1998). Between 1985 and 1993 annual production and planting of loblolly pine bare-root seedlings across the South ranged from 1 to 1.5 billion (Schultz 1999).

Conifer SE has been demonstrated for many genera and species (Tautorus et al. 1991 and Atree and Fowke 1993). Typically, conifer SE proceeds through four steps: initiation, multiplication, maturation, and germination. A fifth step of cryogenic storage may be added when storage of embryogenic cultures is desired. Large-scale production of somatic embryos for operational plantings has been achieved for some genotypes of spruce and fir species (Sutton et al. 1997, Atree et al. 1993, Handley et al. 1994).

The first report of SE in loblolly pine occurred in 1987 (Gupta et al. 1987). Since then several reports have focused on loblolly pine embryo maturation (Uddin et al. 1990, Becwar and Pullman 1995, Li et al. 1997, 1998, and Tang 1998) along with abundant patent activity (Uddin 1993, Pullman and Gupta 1991, Rutter et al. 1998a, 1998b). A few laboratories have reported regeneration of loblolly pine plantlets through SE, but the process is inefficient and embryo quality is unsuitable for operational use (Pullman et al.

1994, Becwar et al. 1995).

Several factors currently limit commercialization of SE for loblolly pine, including low initiation rates (many desirable genotypes are recalcitrant), low culture survival, culture decline causing low or no embryo production, and inability of somatic embryos to fully mature resulting in low germination rates and reduced vigor of somatic seedlings. When this research started, maturation of loblolly pine somatic embryos was rare; resulting cotyledonary embryos were of low quality and most often would not germinate. Maltose, either alone or in combination with glucose, along with ABA, was reported to produce cotyledonary embryos using an MSG salt base (Uddin et al. 1990, Uddin 1993). This report was the staring point for the maturation research reported here. A preliminary portion of this work was presented in Pullman and Webb (1994) and Pullman et al. (1998).

Materials and Methods

Media Preparation, Quality Control, and Culture Conditions.

Medium pH was adjusted with KOH and HCl after the addition of all ingredients except gelling agent and filter-sterilized materials. Gelling agent was added prior to autoclaving at 121°C for 20 minutes. Aqueous stock solutions of L-glutamine or filter-sterilized materials were added to medium cooled to approximately 55°C.

Both osmolality and pH of fresh media were measured as a method for media quality control and to quantify the effect of these variables on maturation. Medium osmolality was measured with a Wescor 5500 Vapor Pressure Osmometer. Seven-mm filter paper discs (Wescor, SS-033) were placed on the medium surface and allowed to equilibrate for at least 10 minutes. Saturated discs were rapidly transferred to the Vapor Pressure Osmometer sample holder and osmolality determined. Two samples were measured and averaged. If measurements did not agree within 10 mmol/kg, a third or fourth measurement was made until two measurements were 10 mmol/kg or less apart.

Embryogenic Tissue Initiation and Maintenance

Loblolly pine cultures were initiated as described by Pullman and Johnson (2002) on medium 505 (Table 1) or modifications of 505. A modification of BM (a modification of ½ P6 salts, Gupta and & Pullman 1990) showed the most structures resembling zygotic embryos and was chosen for use with liquid cultures (Medium 16, Table 1).

Embryogenic cultures of loblolly pine were maintained on media 16 or 16 semisolid with 2.5 g/L Gelrite. Cultures were maintained on gelled medium after initiation and once enough tissue developed were grown in liquid medium. Osmolality for medium 16 was approximately 160 mmol/kg. During the first few weeks of shake culture the embryogenic tissue clumps broke up. Individual cells and small clusters of cells formed. Culture flask volumes were built to a maximum of 5 mL of settled cells plus 45 mL of liquid maintenance media per 250 mL flask or 30 mL settled cells plus 270 mL medium in a 1-liter flask.

Embryogenic cell suspensions were established by adding 0.95-1.05 gram(s) of 10-14-day-old semisolid-grown embryogenic tissue to nine mL of liquid maintenance media in a 250 mL Erlenmeyer flask. Cultures were then incubated in the dark at 20-22°C and rotated at 120 rpm. After 5-7 days each flask was manually swirled to facilitate breakup of the tissue clumps. Seven days after starting, 10 mL of liquid medium was added to the culture flask. At the end of another seven days flask contents were poured into sterile centrifuge tubes and settled for 20 minutes. Old liquid was removed, settled cell volumes were measured, and cells were resuspended in medium at a density of 1 mL settled cells / 9 mL medium. Cultures were then rotated at 90-100 rpm and maintained on a weekly transfer schedule at the same ratio of cells to medium.

Development and Maturation Tests

Ten mL of maturation medium were prepared and poured into 6x1.5-cm Falcon #351007 petri plates. When the media were cool and gelled, sterile 4.25-cm black filter paper discs (Ahlstrom Filtration, No. 8613-0425) were placed on the media surface in the center of the dish. Early-stage embryos, grown in medium 16, were settled for 20 minutes and spent media above the settled cells were removed. Settled cells were gently stirred to mix settling layers, and one-mL aliquots of settled cells were pipetted and spread onto the black filter paper. If cell suspensions were clumped, cells were manually spread over the filter paper surface with a clean sterile forceps. (Note: In 1999/2000 the black filter paper manufacturing process changed and this product caused pH changes detrimental to loblolly pine somatic embryo production.) Black cotton (100%) fabric (Beechwood Country Class Solid 6785) is now substituted for the filter paper. Four to five replications for each treatment-genotype combination were plated. Plates were wrapped in two layers of Parafilm and incubated at 23-25°C in the dark. Cells were subcultured monthly by transferring the support with cells to fresh development medium. After three monthly exposures to fresh medium, embryo production observations were recorded.

Germination of Somatic Embryos

After 2.5-3 months on maturation medium, somatic embryos were selected that exhibited normal embryo shape. Ten embryos were placed horizontally on 20-mL germination medium 55 (1/2 MS salts with 2.5 g/L activated carbon, Table 1) or medium 397 (medium 55 with copper sulfate adjusted to 0.25 mg/L to compensate for copper adsorption by activated carbon) contained in 100x20-mm petri plates. Plates were incubated for 7 days in the dark and then placed under fluorescent light. After 6-7 weeks in the light the embryos were scored for the presence of roots and shoots. An embryo was considered to have germinated when it contained both a root and a shoot.

Conversion, Acclimation, and Field-Testing

Loblolly pine artificial planting soil, provided by Union Camp Corporation, was hydrated at a ratio of 1:1 water to soil. Tall magenta boxes were filled with 45 g of moistened soil and autoclaved. Boxes were opened and allowed to air out overnight in a sterile laminar airflow hood to reduce potential toxic material produced by heating peat moss contained in the soil mix. Soil was remoistened to replace evaporated water and loblolly pine germinants with a root and at least 1 cm of shoot growth were planted, one plant per box. At the time of planting and every two weeks thereafter, 2.0 mL of ¼-strength Schenk and Hildebrant (1972) salt solution was added as fertilizer. Containers were incubated under 16 hours of fluorescent light daily at about 48 foot-candles. After seedlings showed epicotyl growth for two to four weeks, they were carefully transferred to leech tubes containing the same soil. Leech tubes were enclosed in nonsterile plastic bags containing a wet polyester pad to moisten the air. Over a period of several weeks the bags were opened for longer periods each day, first under fluorescent lights and later in a greenhouse, until no epicotyl wilting was observed and the bag remained open. Then the bag was removed and the seedlings grew normally in the greenhouse environment.

After one year in the greenhouse, 35 seedlings (genotype 195, initiated in summer 1994 from tree UC10-1003, matured on medium 240) were over-wintered in a greenhouse with natural light where temperatures were not allowed to go below freezing. In late January 1997, plants were planted in a field plot at the Union Camp Ogeechee Forest in Tattnall County, Georgia. The spacing between seedlings was 10x6 feet. For the next two growing seasons plant survival and height were tracked.

Cryogenic Storage and Culture Retrieval

Embryogenic suspension cultures were grown in medium 16. Five to seven days after subculture, 30 mL of settled cells were added to 120 mL of medium 16

supplemented with 0.2M sorbitol and grown for 24 hours. Cells were settled and subcultured to 16 supplemented with 0.4M sorbitol for another 24 hours. Cells were again settled, 53 mL of liquid removed, and the 97 mL remaining were placed into a one-liter flask; and 1.2 mL of filter-sterilized dimethyl sulfoxide was added slowly. The mixture was cooled on ice for 15 minutes before dispensing an additional 1.2 mL of DMSO. Fifteen minutes later a final aliquot of 1.1 mL of DMSO was added to bring the cryoprotectant concentration to 5%. 1.8 mL of the suspension/cryoprotectant mixture was pipetted into 2 mL Nalgene cryogenic vials and placed into a programmable freezer. Vials were cooled to –35°C at a rate of 0.33°C per minute. Vials were then transferred to a cryobox and submerged in a liquid nitrogen storage chamber for long-term storage.

To retrieve cultures, vials were removed from the liquid N_2 and thawed in a 37°C water bath for 5 minutes. Vials were opened, flamed to sterilize the vial lip, and contents were poured onto a sterile black filter disc (4.25 cm) on a petri dish (100x15 mm) containing 20 mL semisolid medium 16 + 2.5 g/L Gelrite. After one hour the filter paper overlain with cells was moved to a plate of fresh medium. Eighteen hours later cells were again transferred to gelled 16. Plates were in kept in the dark.

Evaluations of Somatic Embryo Quality

Somatic embryos from medium 240 appeared morphologically similar to zygotic embryos at stage 8-9.1 (Pullman and Webb 1994). To confirm our observations, comparisons between somatic and zygotic embryos were made for dry weight, water content, germination, and gene expression patterns.

Zygotic and Somatic Embryo Fresh, Dry Weight, and Water Content. Open-pollinated loblolly pine cones were collected weekly through the course of seed development for two seed-orchard-grown trees, UC5-1036 from Union Camp Corp. (now International Paper) at Belville, GA, and BC-1 (S4PT6) from Boise Cascade Corp. at Lake Charles, LA. Cones were shipped on ice to IPST, received within 24-48 hours, and stored at 4-5°C for 1-2 weeks. Upon arrival and prior to use in experiments samples of seeds were evaluated for embryo stage (Pullman and Webb 1994). Each week 10-20 embryos were isolated from cones from each tree, staged, and collected to obtain fresh and dry weights and moisture contents. To minimize error from tissue drying, embryos were isolated in a moist chamber, enclosed in small preweighed aluminum weighing containers, and weighed on a five-place metric balance. Embryos were dried overnight at 70°C and reweighed to obtain dry weights. Data were obtained from embryos for each of four cones for each collection time and mother tree. From this data moisture content, ovule fresh weight, and ovule dry weight could be calculated. The same process was

followed with somatic embryos from replicate plates of maturation medium 240.

Zygotic Embryo Germination. Weekly cone collections containing stages 5-9.2 were used to test germination. Seeds were isolated and sterilized as described by Pullman and Johnson (2002). After sterilization and rinsing, seeds were cracked using a hemostat, pried open with a scalpel, and integument, nucellus, and female gametophyte (megagametophyte) removed. About 50-120 embryos were tested per developmental stage with a maximum of 10 embryos per plate of germination medium 397.

Zygotic and Somatic Embryo Gene Expression: Arraying cDNA Clones and Probe Preparation. cDNA clones of embryo-expressed genes were isolated as described previously (Xu et al. 1997, Cairney et al. 1999). PCR was performed using plasmid DNA to amplify the insert region. Five μg of PCR product in 15 μl were mixed with 3.3 μl 3.0 M NaOH and incubated at 65°C for 30 min. After cooling to room temperature, 20.5 μl 20xSSPE (containing 5% gel loading dye) was added to the denatured DNAs which were then blotted onto Hybond N+ membranes (Amersham) as arrays using a VP 386 pin blotter (V&P Scientific, Inc., San Diego, CA). Each DNA was dot-blotted four times as a quadrate on the membrane. The dots are ~1.2 mm in diameter and each of them contains about 30 ng DNA. The DNA was then cross linked to the membrane at 120,000 mJ/cm2 in a CL-1000 UV-linker (Upland, CA) prehybridized with hybridization buffer (0.5 M Na-phosphate, pH7.2, 5% SDS, and 10 mM EDTA) at 65°C for 30 min. Probes were made by Advantage PCR (Clontech) using the first strand cDNAs generated by SMART cDNA synthesis kit (Clontech) as templates. The 50 μl PCR reaction mix contained 1x PCR buffer, 5 μl dATP+dGTP+dTTP (5 mM each), 1 μl T21VN primer (10 μM) , 1 μl SCSP oligo (5'-ctcttaattaagtacgcggg-3', 10 μM), 5 μl template (first strand cDNA), 1 μl KlenTag enzyme mix, and 5 μl ^{32}P-dCTP (Amersham). The cycle conditions were 94°C 2 min, 15 cycles of 95°C 15s, 52°C 30s, 68°C 6 min. The PCR products were purified using NICK column (Pharmacia) according to the manufacturer's instructions. Hybridization was performed in 3 mL hybridization buffer in a hybridization oven at 65°C overnight. The membrane was then washed three times with 0.1x hybridization buffer at room temperature for 10 min and once at 42°C for 30 min. The autoradiograph images were scanned and digitized using GelPro 3.0 (Media Cybernetics, MD).

Results and Discussion

Embryo Staging. The staging system of Pullman and Webb (1994) was used to evaluate morphological development in zygotic and somatic embryos (Figure 1). Stage 9 zygotic

embryos were also categorized by the week they were collected: 9.1 (stage 9, week 1), 9.2 (stage 9, week 2), etc. This system is based on the literature concerning embryology within the pine family and is detailed to enable close scrutiny of embryo development. We use the system for classification of both zygotic and somatic embryos to compare early, mid, and late-stage development. This system was useful to help understand variation due to location and time of zygotic embryo development as well as variation in culture due to genotype.

Embryogenic Tissue Initiation and Maintenance.

Loblolly pine cultures were initiated as described by Pullman and Johnson (2002) on medium 505 or modifications of 505. Early experimentation tested several salt bases including DCR (Gupta and Durzan 1985), MSG (Becwar et al. 1988), and ½ P6 modified (Gupta and Pullman 1990). The ½ P6 modified showed the most structures resembling zygotic embryos and was chosen for use with liquid cultures (Medium 16, Table 1).

Maintenance.

Twenty-five initiated cultures that contained enough embryogenic tissue for starting liquid cultures were grown in medium 16 and characterized for early-stage embryo morphology (Table 3). Microscopic examination of cultures showed differing morphologies varying from mostly single cells or clumps to well-formed somatic embryos which resembled early zygotic embryos at stages 1-3 (Figures 1, 2A). Out of 25 new initiations tested, 18 (72%) were able to start liquid cultures. Two of the seven cultures that did not start were retested for their ability to start a liquid culture. Both of these cultures started liquid cultures, bringing the overall liquid culture success rate to 80%. The remaining five cultures had stopped growing in the maintenance medium and were no longer alive, suggesting that establishment in liquid medium 16 shortly after initiation may prevent or delay the loss of a culture. Average weekly multiplication rates over five weeks ranged from 1.5 to 4.7 fold per week. Each week several drops of embryo suspensions for each culture were examined and scored for the most advanced embryo stages present. Average weekly embryo stage ratings varied from 1-2.9. Maximum stage ratings of at least 2 were seen in 16/18 (89%) of the cell suspension cultures that started in the first attempt. The maximum stage rating is important because previous observations showed that liquid cultures with stage ratings of two or higher had the best chance of producing cotyledonary embryos. Approximately 25% of the genotypes in liquid culture consistently showed the presence of organized structures at stage 2 or better.

The observation that only liquid cultures with organized early-stage embryos

(stages 1.5-2) produce cotyledonary embryos suggests that stage-related criteria must be met before embryo development will continue. In addition, this observation suggests that we focus research on obtaining organized structures in unresponsive cultures and avoid placement of unorganized cultures into the next developmental step of the protocol. Other researchers have also reported stage-related responses such as for abscisic acid (ABA) during conifer embryo development (Jalonen and Von Arnold 1991).

Embryo Development and Maturation Experiments

MM6 Screening. Fifty-three genotypes, maintained on medium 16 + 2.5 g/L Gelrite, were tested for cotyledonary embryo production on MM6 medium, the 6% maltose/ABA-containing maturation medium of Uddin (1993) (medium 24, Table 1). For each genotype, four embryogenic tissue masses about 1 cm in diameter were placed on a 100x15-mm petri plate containing 20 mL of maturation medium. Transfers to new medium were carried out every two weeks for four transfers. At the end of three months, cultures were observed for presence of cotyledonary embryos. Only two of 53 genotypes tested produced cotyledonary embryos. The few embryos produced were of low quality, appearing short and barrel-shaped, and were embedded in tissue. These embryos were not able to germinate when placed on medium 55.

PEG Replacement of Maltose. Results from screening 53 genotypes on medium 24 showed promise for embryo production but required significant improvements in embryo yield and quality. Polyethylene glycol (PEG) had been reported to improve embryo yield and quality in both white spruce (Atree et al. 1991) and Douglas fir (Gupta et al. 1993). We began testing by substituting 2 and 4% of the maltose with 7.5 and 13% PEG respectively, while maintaining osmolality at ~240 mmol/kg. Five replications for each of three genotypes, grown in medium 16, were plated onto maturation medium 24, 226 (maltose reduced to 4% and 7.5% PEG molecular weight 8000 substituted as an osmoticant), or 225 (maltose reduced to 2% and 13% PEG substituted as an osmoticant).

In this work an effort was made to measure osmolality of maturation media under test and to compare media with similar osmotic potential. Both maltose and PEG were varied. To help identify the correct combinations of maltose and PEG to obtain target osmotic potential near 240-250 mmol/kg, we used a plot of medium osmotic potential vs, % PEG (8000) added to basal medium containing 2% maltose. A highly significant curvilinear relationship (r^2 = 0.964) was obtained for % PEG vs. medium osmolality (mmole/kg). By moving along the curve to the osmolality of the basal without PEG, we were able to predict osmolality increase that a defined addition of PEG would

cause. By using this relationship, the target osmolality was obtained for 6% maltose/0% PEG, 4% maltose/7.5% PEG, and 2% maltose/13% PEG.

Substitution of maltose with PEG showed a clear benefit in embryo development. All three genotypes tested did not produce cotyledonary embryos in medium 24. When 2% of the maltose was replaced with 7.5% PEG, many embryos developed to stages 3-4 faster, producing large bullet-shaped embryos (Figure 1). At stages 5-6 most embryos callused and stopped development. A few short barrel-shaped cotyledonary embryos formed that were covered by callus on the sides and base. When 4% of the maltose was removed and substituted with 13% PEG, embryos developed further, emerging from the callus and increasing yield slightly. The three media tested had similar osmolality measurements ranging from 227-233 mmole/kg. When tested for germination on medium 55 (Table 1), none of the embryos were able to produce a root and shoot.

Based on results from this experiment, a second test included 29 genotypes that were grown in medium 16 and plated with two to five replications on medium 225. Of 29 cultures plated on 225 (13% PEG, 2% maltose), one culture was contaminated, and five out of 28 produced cotyledonary embryos. Four of the five cultures produced less than five cotyledonary embryos per mL settled cells.

In a third experiment, four different molecular weight polyethylene glycol types were tested for cotyledonary embryo production. The PEG mw and (% added to medium) were as follows: 10,000 (13%), 8,000 (13%), 3,350 (10.5%), and 1,450 (8.5%). Four replications of 1-mL aliquots of cells were plated for each PEG type using four loblolly pine genotypes. The four PEG mw types, 8,000, 10,000, 3350, and 1450, produced average medium osmolality readings of 251, 249, 244, and 251 mmol/kg and average cotyledonary embryo yields per mL of plated cells of 10.6, 2.6, 1.3, and 0.3, respectively. The highest embryo yields for all four genotypes occurred on PEG mw 8,000, significantly greater, P=0.5, than on all other PEG molecular weights.

MSG vs ½ P6m Salts and Maltose vs Maltose/PEG. Maturation medium 24 is based on MSG salts. However, embryos showed good growth in the initiation and maintenance steps using medium containing modified ½ P6 salts and case amino acids. Thus, ½ P6m salts were compared with MSG salts in maturation media in combination with 0, or 13% PEG with 6, or 2% maltose, respectively. Four genotypes were tested with four replications per genotype and treatment.

Medium osmolality ranged from 246 to 259 for test media. One genotype did not form any cotyledonary embryos on any test medium. All of the other genotypes tested showed high yields of cotyledonary embryos in medium 240 (½ P6m salts, 2% maltose, 13% PEG). Embryo yield and quality clearly showed an improvement with the

combination of modified ½ P6 salts and PEG. In addition to higher yields, cotyledonary embryos developed faster and had a smoother surface (less callus) and appeared more like zygotic embryos in shape showing distinct root, hypocotyl, and cotyledon regions (Figure 2B). Statistical analysis of data from the three cotyledonary embryo-producing genotypes showed no significant differences in normal or total cotyledonary embryos when MSG salts were compared to ½ P6m salts with 6% maltose. However, in the presence of 13% PEG, ½ P6m produced greater yields of normal and total embryos compared to MSG (P=0.05). PEG (13%) resulted in a statistically significant increase in cotyledonary embryos (P=0.05) when tested in MSG or ½ P6m salts. Figure 1 shows the somatic embryo stages visible during development on medium 240 along with zygotic embryos at similar stages.

Screening Genotypes for Maturation in Medium 240. Twenty of 25 new cultures, grown in medium 16 for at least five subcultures, were plated onto medium 240 (Table 3). Embryos from most genotypes became visible to the naked eye during the second and third subcultures on maturation medium. Fourteen of 25 genotypes (56%) produced cotyledonary somatic embryos morphologically similar to zygotic stages 6-9.1. Genotypes with stage 7-9.1 embryos and with suitable numbers of embryos were tested for germination on medium 55. Four out of the seven genotypes tested showed 1-33% germination. Nineteen of the liquid-grown cultures were placed in cryogenic storage.

Maturation Improvement. The combination of 2% maltose, 13% PEG 8000, and ½ P6 modified salts resulted in a major improvement in embryo yield and quality. The mechanism of improved embryo development may be found in studies of the osmotic environment in developing loblolly pine ovules. Pullman (1997) measured water potential of developing loblolly pine ovules. As embryos developed from stage 2 to 4-6, ovule water potential dropped 100-200 mmole/kg to 200-300 mmole/kg. Sucrose has been reported to break down in conifer maturation medium into glucose and fructose, causing medium osmolality to increase (Iraqui and Tremblay 2001). Sucrose hydrolysis in the medium has been attributed to cell wall invertase (Yu et al. 2000). Use of sucrose during stages 4-6 causes osmolality to rise while maltose allows osmotic levels to remain static or slowly decline (unpublished data). We speculate that maltose is a superior carbohydrate/osmoticant because it facilitates creation of a more natural osmotic environment during the critical developmental period of stages 4-6.

Li et al. (1997, 1998) also used Uddin (1993), Pullman (1994), Gupta and Pullman (1991), and Pullman and Gupta (1991) as a base to research maturation improvements in loblolly pine through the use of maltose and PEG. They concluded that PEG alone and maltose/PEG improved embryo maturation for work with two loblolly

pine genotypes. However, no germination assessment was provided. Their maturation protocol differs significantly from ours by containing agar as a gelling agent, combining high levels of ABA (10-40 mg/L) and activated carbon (1.5 g/L), and using lower levels (5-10%) of a lower molecular weight PEG (3500). In our case the combination of maltose PEG (8000) and Gelrite (2.5 g/L) produced a superior somatic embryo. Patent coverage (Pullman et al. 1991) of the use of combinations of ABA and activated carbon prevented us from exploring the use of ABA and charcoal in conifer maturation medium.

Germination of Somatic Embryos

Observations of germination tests with ten different genotypes showed that somatic embryos respond during the first 4-5 weeks by expanding the hypocotyl followed by expansion of the cotyledons. During the next 4-5 weeks apical primordia appeared and slowly expanded into needle growth. After approximately two months, root growth began (Figure 2C). Both root and shoot growth continued slowly. While the above sequence of germination is slow and awkward when compared to normal seed embryos, the ability to germinate somatic embryos repeatedly from several genotypes produced on medium 240 was encouraging and provided us with a baseline for future improvements.

Conversion, Acclimation, and Field Testing

Seedling conversion and growth was highly related to the quality of the germinant at the time of planting. Germinants with larger shoots, longer, straighter hypocotyls, and to some extent longer roots fared best. At first germinants appeared to spend several weeks in an acclimation period. Initially, germinants were a dull, dusty-green color with no growth of the epicotyl or hypocotyl noted. After 2-4 weeks, new epicotyl growth formed as bright shiny needles growing upward out of the initial shoot. This phase lasted for 1-2 weeks. The epicotyl then ceased growing and after 1-2 weeks began growing again as bright green needles that spread out. At this stage, seedlings were gently transferred to leech tubes in nonsterile transparent plastic bags that were gradually opened to the culture room and later greenhouse environment. Across all germinants, 30/59 (51%) survived conversion and acclimation and continued growth in the greenhouse (Figure 3AB). Higher survival occurred with improved germinant quality.

In October of the planting year all 35 field-grown trees from somatic embryos had survived and averaged 1.9 feet in height. Trees again showed 100% survival and averaged 5.2 feet in the second year. For reference, 20 nearby traditionally generated loblolly pine seedlings that were planted at about the same time had an average height of 8.6 feet. It is speculated that the differences in height may be due to a slow start in the

first growing season from somatic seedling residency in our greenhouse for one year.

Cryogenic Storage and Culture Retrieval

Growth first appeared after three weeks to two months as translucent finger-like tissue projecting from stored embryo clumps. When the tissue mass reached a diameter of 2 mm, retrieval was considered successful and tissue was further maintained in 16 or on gelled 16. When three vials per culture were retrieved from cryogenic storage, 18/19 (95%) of the cultures stored were successfully revived.

Comparisons of Zygotic and Somatic Embryo Quality

Numerous parameters have been used to evaluate embryo quality including morphology, weight, water relations, biochemical contents, gene analyses, and germination kinetics. Here we compare zygotic embryos at different stages of development and our most advanced cotyledonary somatic embryos using several measures of embryo quality.

Zygotic and Somatic Embryo Fresh, Dry Weight, and Water Content. Zygotic and somatic embryo dry weights and water contents are shown in Table 4. Zygotic embryos accumulated dry weight and decreased percent of water content with each advance in embryo stage. Somatic embryos from genotypes with the most advanced development resulting from 11-13 weeks of growth on medium 240 ranged from 0.3 to 0.9 mg dry weight per embryo. Somatic embryo morphology and dry weights most resembled zygotic embryos at stages 8-9.1.

Zygotic Embryo Germination. Mature zygotic embryos germinate when the root emerges before or coincident with the shoot. In contrast, somatic embryos germinate in reverse sequence with the cotyledons greening first, shoot emergence, and then much later, if at all, the root appears. To determine when immature zygotic embryos acquire the capacity for normal germination, we isolated embryos at stages 5-9.2 and placed them on germination medium. From this functional study of zygotic germination, we were able to determine when immature zygotic embryos become competent to germinate. It also allows us to compare germination for zygotic vs, somatic embryos, providing estimates of somatic embryo developmental stage. The results indicate that our somatic embryos develop to stages 7-8. This suggests that early somatic embryo development, stages 1-6, occurs normally, and our block is in the later stages of embryo development.

Most embryos survived the sterilization and isolation process with 80 and 100% survival, respectively, for stage 5 and 9.2 for UC5-1036 embryos. Abnormal cotyledon development (fewer cotyledons or with altered morphology) in UC5-1036 was 40% for

stage 5 embryos, about 15% for stages 7-9.1, and 5% for stage 9.2. It was interesting to note that 60% of the zygotic embryos at stage 5 were able to produce normal cotyledons on germination media. 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. Hypocotyl growth increased with embryo maturity. Stage 5 hypocotyls did not elongate during germination tests whereas stages 7-9.1 showed increasing elongation with advancing stage. About 50% of the stage 5 embryos formed a shoot meristem that developed into a shoot, indicating that a functional shoot apical meristem is formed at or before stage 5. Few stage 5 or stage 6 embryos formed a root, suggesting that functional root development begins at stage 7. Why only 50% of the embryos formed a functional shoot is unclear. The percentage of embryos that formed a root and average root length increased as stage increased past 7. Shoot production, root production, and germination (both shoot and root production) for UC5-1036 and S4PT6 cone collections at stages 5-9.2 are shown in Figure 6. Both embryo collections started germinating at stage 7-8, and the germination percent increased with stage. This observation suggests that zygotic embryos develop the capability to germinate at stage 7-8. In comparison, zygotic embryos at stages 7-8 and our most advanced somatic embryos showed similar germination performance in both sequence and percentage of germination.

A functional transition appears to occur in zygotic embryos between stage 8 and 9.1 for germination. 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. 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. Stage 8 had only slightly more embryos that formed only shoots versus ones that formed only roots or that germinated. This suggests that in embryos at stage 9.1 and beyond, the root meristem is activated first upon germination. This is the natural sequence for mature zygotic embryo germination from seed. Thus before development has reached stage 7, neither zygotic nor somatic embryos have the capacity to germinate in the root-shoot sequence observed for fully mature zygotic embryos. In maturing embryos, a developmental transition occurs in the ability of the root or shoot to form first during zygotic embryo germination.

Zygotic and Somatic Embryo Gene Expression. Somatic embryos appear to develop normally through stages 1-8 and then stop further growth. Somatic embryos at the end of development are often similar to zygotic stage 8 embryos, having similar morphology and dry weights between stages 8-9.1 and comparable germination sequence

to zygotic embryos at stages 7-8. To further support these observations, we compared gene expression profiles during somatic and zygotic embryo growth (Cairney et al. 1999, 2000). If somatic embryos are at stage 7-9.1 they should show similar gene expression activity to that seen in corresponding zygotic embryos. Gene expression patterns for 326 differentially expressed cDNA fragments were determined across the sequence of somatic and zygotic embryo development (Cairney et al. 1999). For each gene, expression activity for our most advanced somatic embryos, estimated to be at stage 9.1, was divided by expression activity for that gene in zygotic embryos at stages 1 through 9.4. A ratio of 1 indicates that mRNA level for a given gene is identical in both types of embryo, a ratio greater than 1 indicates that mRNA level is higher in the somatic embryo than in the zygotic embryo to which it is being compared, and a ratio less than 1 indicates higher zygotic embryo expression. The resulting ratios of gene activity were categorized by degree of difference: >5.0, 2.0-4.99, 0.5-1.99, 0.2-0.49, and <0.2, and the percentage of genes falling into each category was calculated and plotted (Figure 5). As expected, gene expression of stage 9.1 somatic embryos is dissimilar to gene expression in early or late stage zygotic embryos. However, when the most mature somatic embryos were compared to zygotic embryos from stage 5 through 9.1, the number of genes expressed to similar degrees increased with stage. Gene activity for somatic embryos estimated to be at stage 9.1 were most similar to gene activity in zygotic stage 7, consistent with our previous conclusion that our most advanced somatic embryos are similar to stages 7-9.1.

Conclusions

While commercial application of SE technology may not require full-term embryo maturity to produce high-value somatic seedlings, significant advancement in embryo maturation is required. Maturation improvement represents a significant challenge. Few, if any, somatic embryo systems produce embryos similar in final size or stage, biochemistry, or vigor when compared to zygotic embryos. Many embryogenic systems, including conifers, appear to produce somatic embryos that are capable of germination and plant establishment, but that do not fully mature, resulting in slow germination and initial growth. Two approaches may improve germination performance: (1) advancing somatic embryo development and (2) improving the germination process through media, and environmental changes. Both approaches need to be investigated.

Major progress has been made in the areas of initiation, maintenance, embryo development, germination, conversion, and cryogenic storage. However, there are many challenges ahead, particularly in the area of embryo quality. Embryo quality and vigor

must be improved during all stages for the somatic embryogenesis process to become commercial. With the successful storage of cultures in liquid nitrogen shortly after initiation we hope to prevent any culture decline over time, decrease the labor to maintain cultures, and have available a bank of cultures with known histories of performance in the somatic embryogenesis process. Research could then focus on the particular step or embryo stage where improvement is needed.

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Table 1. Media compositions for initiation, maintenance, development, and germination media.

Components mg/L	505	16	24	225	239	240	55
NH_4NO_3	200	603.8	0	0	200	200	206.3
KNO_3	909.9	909.9	100	100	909.9	909.9	1170
KH_2PO_4	136.1	136.1	170	170	136.1	136.1	85
$Ca(NO_3)_2 \cdot 4H_2O$	236.2	236.2	0	0	236.2	236.2	0
$CaCl_2 \cdot 2H_2O$	0	0	440	440	0	0	0
$MgSO_4 \cdot 7H_2O$	246.5	246.5	370	370	246.5	246.5	185.5
$Mg(NO_3)_2 \cdot 6H_2O$	256.5	256.5	0	0	256.5	256.5	0
$MgCl_2 \cdot 6H_2O$	101.7	101.7	0	0	101.7	101.7	0
KCl	0	0	745	745	0	0	0
KI	4.15	4.15	0.83	0.83	4.15	4.15	0.415
H_3BO_3	15.5	15.5	6.2	6.2	15.5	15.5	3.1
$MnSO_4 \cdot H_2O$	10.5	10.5	16.9	16.9	10.5	10.5	8.45
$ZnSO_4 \cdot 7H_2O$	14.69	14.4	8.6	8.6	14.4	14.4	4.3
$Na_2MoO_4 \cdot 2H_2O$	0.125	0.125	0.25	0.25	0.125	0.125	0.125
$CuSO_4 \cdot 5H_2O$	0.1725	0.125	0.025	0.025	0.125	0.125	0.0125
$CoCl_2 \cdot 6H_2O$	0.125	0.125	0.025	0.025	0.125	0.125	0.0125
$FeSO_4 \cdot 7H_2O$	13.9	6.95	27.8	27.8	13.93	13.93	13.93
Na_2EDTA	18.65	9.33	37.3	37.3	18.65	18.65	18.65
Maltose	15,000	0	60,000	2,000	60,000	20,000	0
Sucrose	0	30,000	0	0	0	0	20,000
PEG 8,000	0	0	0	130,000	0	130,000	0
myo-Inositol	20,000	1,000	100	100	100	100	100
Casamino acids	500	500	0	0	500	500	0
L-Glutamine	450	450	1450	1450	450	450	0
Thiamine•HCl	1	1	0.1	0.1	1	1	1
Pyridoxine•HCl	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Glycine	2	2	0	0	2	2	2
2,4-D	0	1.1	0	0	0	0	0
NAA	2	0	0	0	0	0	0
BAP	0.45	0.45	0	0	0	0	0

Kinetin	0.43	0.43	0	0	0	0	0
ABA	0	0	5.2	5.2	5.2	5.2	0
Activated Carbon	50	0	0	0	0	0	2,500
Gelrite	2,000	0	2,500	2,500	2,500	2,500	0
TC Agar	0	0	0	0	0	0	8,000
PH	5.7	5.7	5.8	5.8	5.7	5.7	5.7

Table 2. Medium osmolality and number of normal and total cotyledonary embryos produced per mL of settled cells for each maturation medium.

Medium	Osmolality mmol/Kg	Average Number of Normal (NCE) and Total Cotyledonary Embryos (TCE) Genotype 31		Average Number of Normal (NCE) and Total Cotyledonary Embryos (TCE) Genotype 41		Average Number of Normal (NCE) and Total Cotyledonary Embryos (TCE) Genotype 195	
		NCE / mL	TCE / mL	NCE / mL	TCE / mL	NCE / mL	TCE / mL
24	257	0.3	0.5	0	1.0	2.5	8.0
225	253	1.0	1.0	0	0.5	151	161
239	246	0	0.5	0	1.5	6.0	41.8
240	259	30.8	46.0	0	6.8	225.0	230.8

Table 3. Loblolly pine culture performance: starting liquid cultures, weekly growth rate as settled cell volumes, embryo stage in liquid culture (medium 16), and maximum stage rating in development and maturation medium (medium 240).

Culture #	Origin[3]	Weekly Settled Cell Volume (5 wk avg.)	Liquid Stage Rating - (5-wk average/ maximum rating)	Cotyledonary embryos/mL plated cells, repeated platings	Germination (epicotyl & root growth) on med 55	Cryogenic Survival
245	UC5-1036	2.4	1.7 / 2	2.6, 15.8	Not Tested	3/3
246	UC5-1036	1.6	1.4 / 2	0,0		1/3
247	UC5-1036	1.8	1.7 / 2	1.2, 2.5, 5	Not Tested	3/3
248	BC-3	No Start[2]				
249	WV-F2	3.2	2.0 / 2.5	9.6, 0	Not Tested	3/3
250	WV-F2	3.6	1.7 / 2	0, 0		2/3
251	WV-F2	No Start[1]		0, 23		3/3
252	BC-9	2.7	2.5 / 3	7.8, 50+, 25	Not Tested	2/3
253	UC10-33	4.6	1.7 / 2	0.8	Not Tested	3/3
254	UC10-33	2	1.8 / 2	0, 11	Not Tested	3/3
255	BC-9	1.8	2 / 2	18.6, 12.3, 15	0/70 (0%)	1/3
256	WV-F2	1.3	1.8 / 2	7.4	Not Tested	0/3
257	UC10-33	3.2	2.1 / 3	8.4, 10.5	1/70 (1%)	3/3
258	UC10-5	No Start[2]				
259	BC-9	2.1	2.9 / 3	39.8, 23.6, 41.6	0/500 (0%)	3/3
260	BC-3	1.5	2 / 2	8.0, 21.2	0/20 (0%)	2/3
261	UC5-1036	1.4	2.2 / 3	40.8, 33.8	16/349 (5%)	3/3
262	WV-I2	1.9	1 / 1	0	Not Tested	3/3
263	UC10-5	No Start[2]				
264	WV-F2	No Start[2]				
265	UC5-1036	No Start[1]				
266	UC10-33	2.3	2.7 / 3	10.8, 1.3	33/100 (33%)	3/3
267	UC10-33	2.4	1.6 / 2	24, 4.5	20/151 (13%)	3/3
268	UC5-1036	4.7	1.5 / 1.5	1.6, 3.7	0/20 (0%)	3/3
269	BC-2	No Start[2]				

Average per step	72% 1st Start 2.5 / 2.2 80% 1st & 2nd Start	Yield per mL cells = 10.6 cotyledonary embryos	6.5% 4/8 germ	18/19 (95%)
Success / 25 Cultures	80%	14/25 (56%)	16%	72%

[1]/ Upon a second try liquid cultures were successfully started.

[2]/ Tissue was unavailable for a second attempt to start liquid cultures.

[3]/ Origin of seed materials used for culture initiation: UC = Union Camp Corporation, BC = Boise Cascade Corporation, WV = Westvaco Company.

Table 4. Zygotic and somatic embryo dry weights and water content.

Zygotic Embryo Stage	Tree S4PT6			Tree UC5-1036		
	Dry Weight (mg)	Standard Error	Ovule Water Content (%)	Dry Weight (mg)	Standard Error	Ovule Water Content (%)
6	0.04	0.01	62	0.07	0.01	--
7	0.08	0.01	--	0.09	0.01	58
8	0.17	0.03	47	0.28	0.04	47
9.1	0.50	0.03	36	0.91	0.07	46
9.2	0.92	0.02	--	1.05	0.12	--
9.8	1.44	0.19	27	1.63	0.07	27

Somatic Embryo Genotype	Dry Weight (mg)	Standard Error	Embryo Water Content (%)
195	0.52		82
260	0.48		76
266	0.63		--
275	0.30		77

Figure 1. Comparison of staged zygotic embryos during natural development and somatic embryos developed on medium 240 over a three-month period.

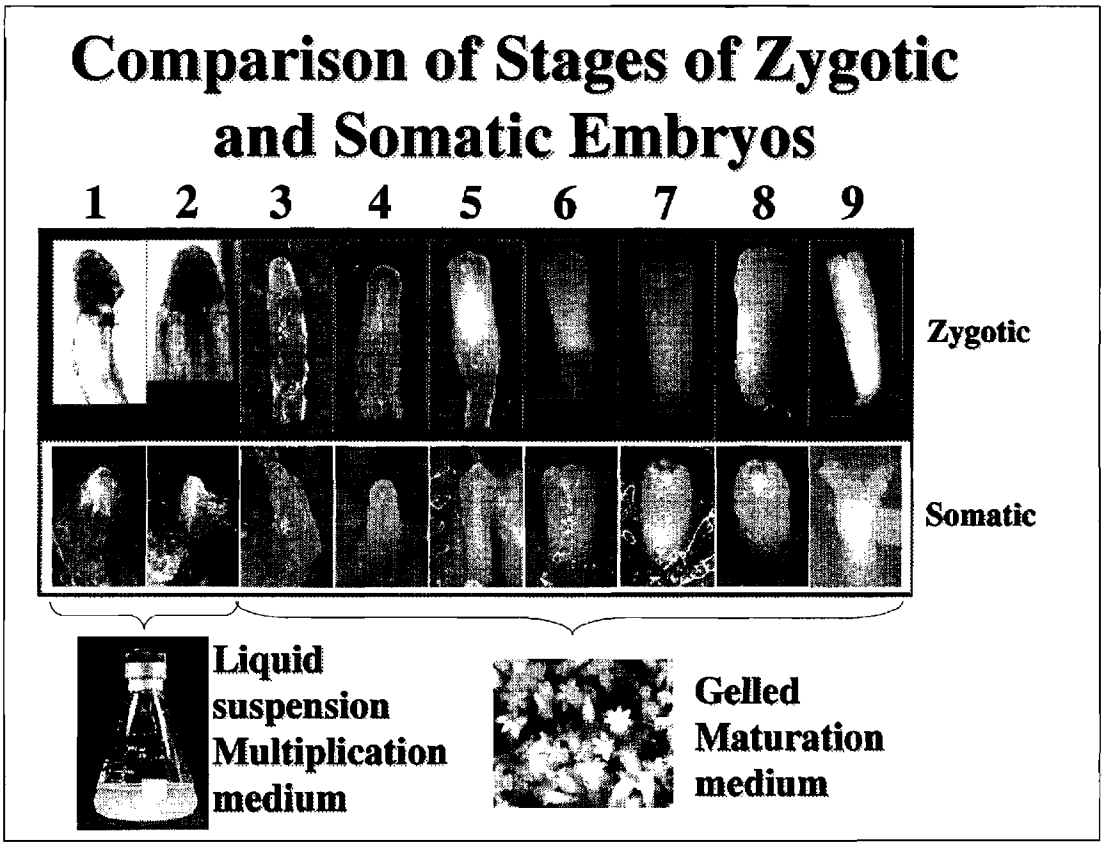


Figure 2. Loblolly pine somatic embryos. (A) Grown in medium 16. Stage 2 somatic embryos are visible. (B) Cotyledonary embryos grown on medium 240. Stage 8-9.1 somatic embryos are visible. (C) Stage 8-9.1 somatic embryos germinated on medium 397 showing root and shoot growth.

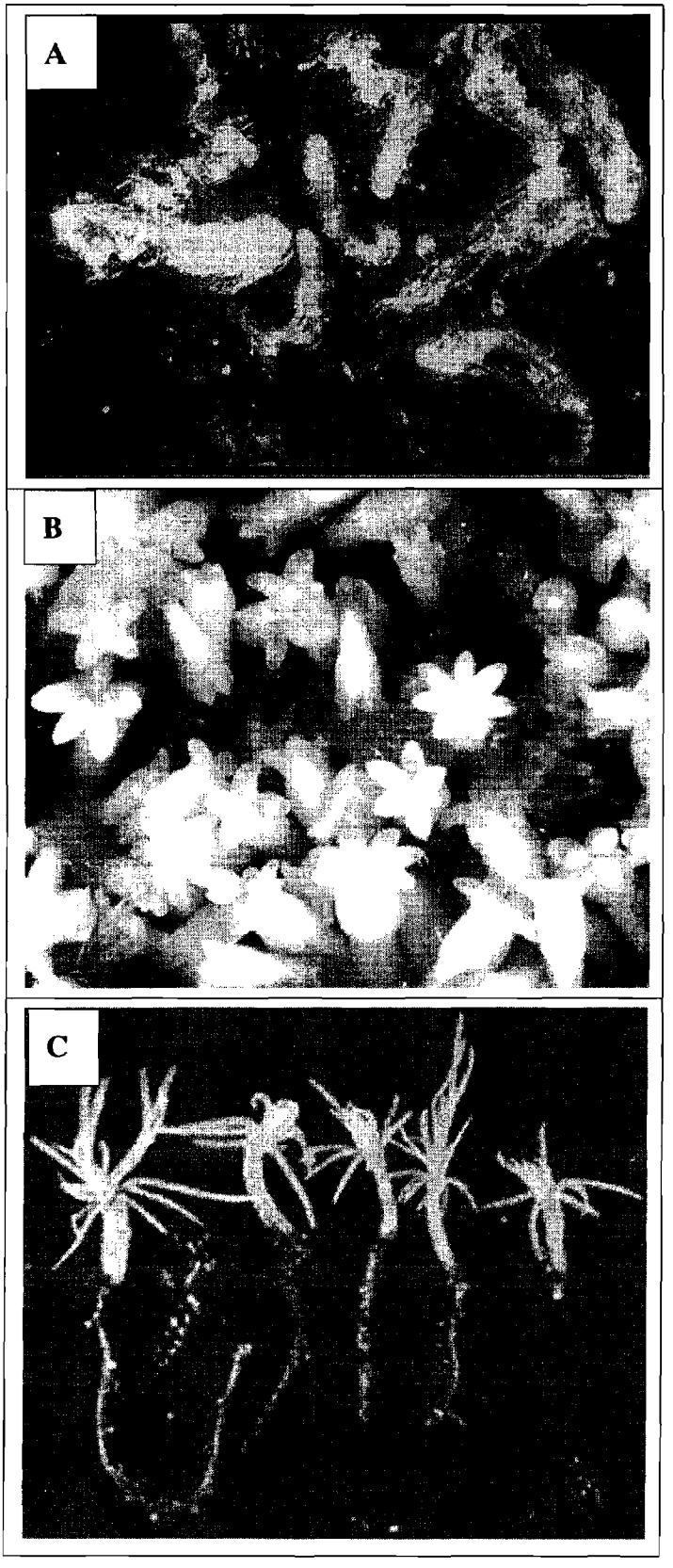


Figure 3. Somatic seedlings of loblolly pine. (A) Acclimated somatic seedling growing in the greenhouse (B) Greenhouse grown somatic seedlings during a spring flush of new growth.

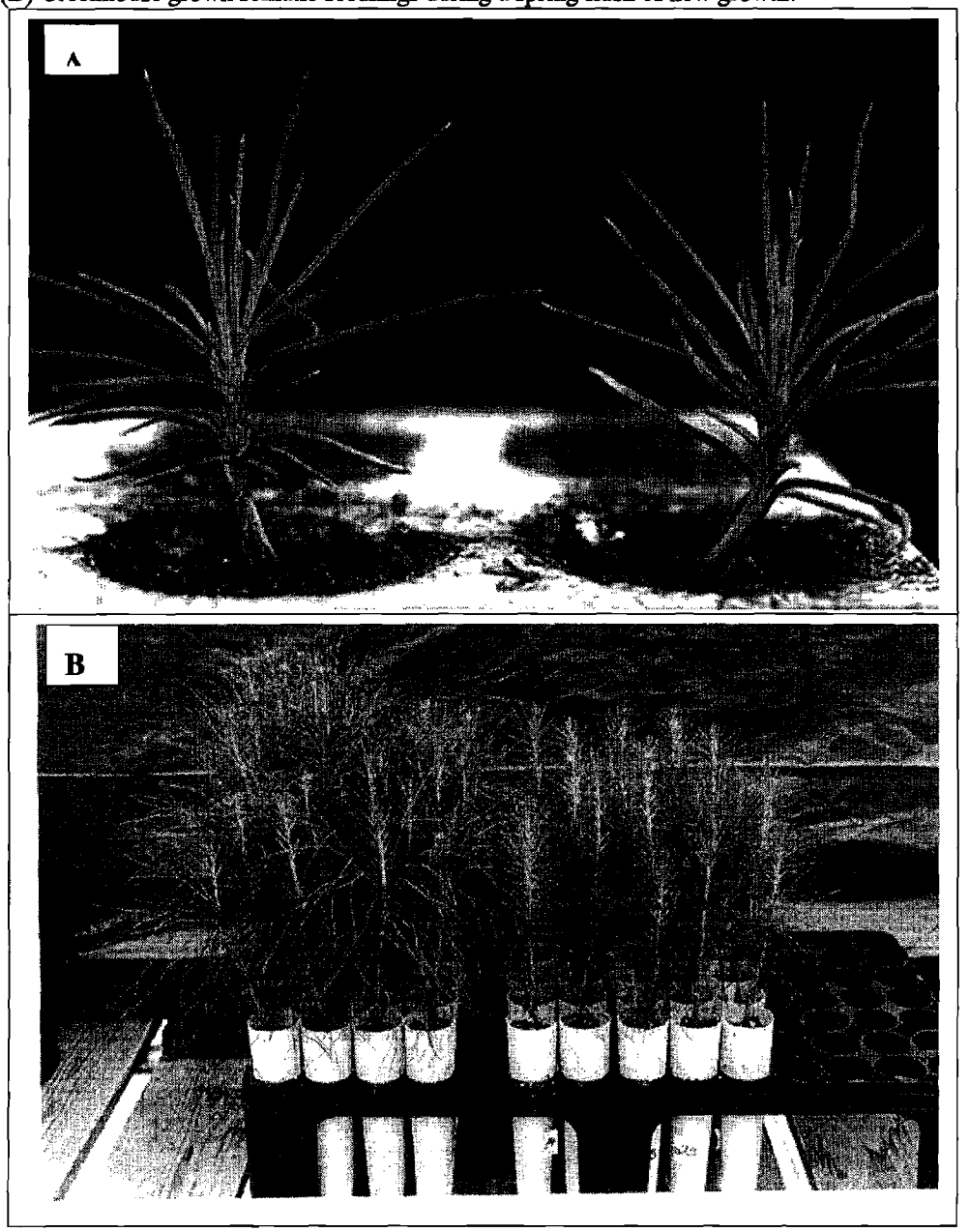
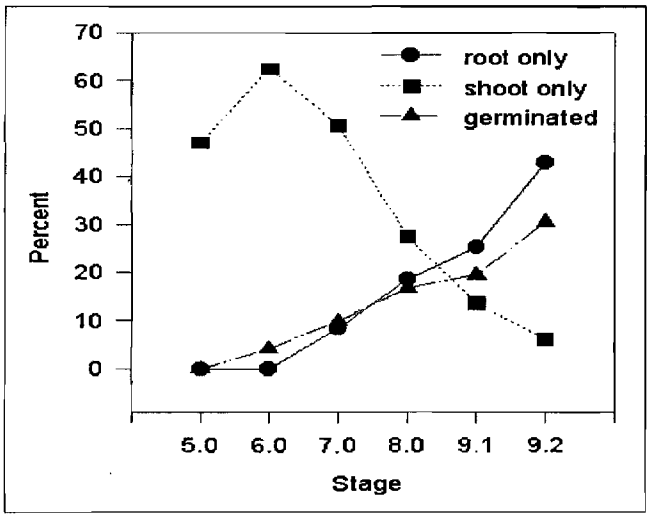


Figure 4. Germination of zygotic loblolly pine embryos isolated at various stages of development. (A) Germination of UC5-1036 stage 5-9.2 embryos obtained in 1997. (B) Germination of S4PT6 stage 7-9.2 embryos obtained in 1998.

A.



B.

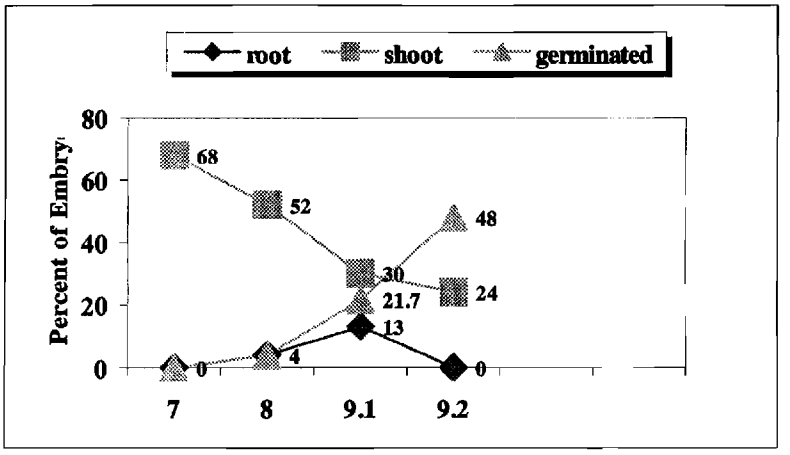


Figure 5. Messenger RNA quantification ratios for stage 9 somatic embryos / zygotic embryos at varying stages. Resulting ratios for gene activity are categorized and percentage of the gene collection is presented by category.