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CELL BIOLOGY AND MORPHOGENESIS

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Improving loblolly pine somatic embryo maturation: comparison of somatic and zygotic embryo morphology, germination, and gene expression

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Abstract Clonal production of loblolly pine (*Pinus taeda* L.) through somatic embryogenesis has the 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 the 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 the development of loblolly pine somatic embryos that can germinate, is presented that combines 1/2 P6 modified salts, 2% maltose, 13% polyethylene glycol 8000 (PEG), 5 mg/l abscisic acid (ABA), and 2.5 g/l Gelrite. A procedure for converting and acclimating germinants to growth in soil and greenhouse conditions is also described. 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 relative to standard seedlings from natural seed. The quality of the resulting embryos was examined and compared to that of zygotic embryos using such parameters as morphology, dry weight, germination performance, and gene expression. All of the observations that were made support the conclusion that even with the new maturation medium somatic embryos grow approximately only halfway through the normal sequence of development and then prematurely discontinue growth.

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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 (McNutt and Rennel 1997). 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 the dominant vegetation on 11.7 million ha and comprises over one-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 Company 1998). Between 1985 and 1993, the annual production and planting of loblolly pine bare-root seedlings across the South ranged from 1 billion to 1.5 billion (Schultz 1999).

Conifer SE has been demonstrated for many genera and species (Tautorus et al. 1991; Attree 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. The large-scale production of somatic embryos for operational plantings has been achieved for some genotypes of spruce and fir species (Attree et al. 1991; Handley et al. 1994; Sutton et al. 1997).

The first report of SE in loblolly pine appeared in 1987 (Gupta and Durzan 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; Tang et al. 1998) along with abundant patent activity (Rutter et al. 1998a, 1998b; Pullman and Gupta

1991; Uddin 1993). 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 and Webb 1994; Becwar and Pullman 1995).

Several factors 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 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 abscisic acid (ABA), was reported to produce cotyledonary embryos by using an monosodium glutamate salt base (Uddin et al. 1990; Uddin 1993). These reports were the starting point for the research reported here. Our objective was to develop a loblolly pine maturation medium that would produce cotyledonary embryos capable of germination. A preliminary portion of the research reported here has been presented in Pullman and Webb (1994) and Pullman et al. (1998).

Materials and methods

Media preparation, quality control, statistical analyses, and culture conditions

Medium was adjusted with KOH or HCl to the pH indicated in Table 1 following the addition of all of the ingredients except the gelling agent and filter-sterilized materials. The gelling agent was added prior to autoclaving at 121 C for 20 min. Aqueous stock solutions of L-glutamine or filter-sterilized materials were added to medium cooled to about 55 C.

Both the 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-millimeter filter paper discs (Wescor, SS-033) were placed on the medium surface and allowed to equilibrate for at least 10 min. Saturated discs were rapidly transferred to the vapor pressure osmometer sample holder, and osmolality was determined. Two samples were measured and

Table 1 Media composition for initiation, maintenance, development, and germination of loblolly pine

Components (g/l)	Medium number:						
	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	20,000	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[a]	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-Dichloroxyacetic acid	0	1.1	0	0	0	0	0
α-Naphthaleneacetic acid	2	0	0	0	0	0	0
6-Benzylaminopurine	0.45	0.45	0	0	0	0	0
Kinetin	0.43	0.43	0	0	0	0	0
Abscisic acid[a]	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

[a]Added as filter-sterilized stock solution after autoclaving.

averaged. If the 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.

Treatments were arranged in a completely randomized design. Data were analyzed by analysis of variance, and significant differences between treatments determined by multiple range test at P=0.05 using STATGRAPHICS PLUS V4.0.

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. The same basal salts used for initiation, a modification of this basal medium (a modification of 1/2 P6 salts, Gupta and Pullman 1990), was used for culture multiplication and maintenance (Medium 16, Table 1).

Embryogenic cultures of loblolly pine were maintained on media 16 or 16 semisolidified with 2.5 g/l Gelrite. Cultures were maintained on gelled medium after initiation, and once enough tissue developed they were grown in liquid medium. The osmolality for medium 16 was about 160 mmol/kg. During the first few weeks of shake culture, the embryogenic tissue clumps broke up into individual cells and small clusters of cells. 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-l flask.

Embryogenic cell suspensions were established by adding 0.95–1.05 g of 10- to 14-day-old semisolid-grown embryogenic tissue to 9 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 7 days, the contents of the flask were poured into sterile centrifuge tubes and settled for 20 min. 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. The 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

A 10-ml aliquot of maturation medium was prepared and poured into 6×1.5-cm Falcon no. 351007 petri plates. When the medium in the plates had cool and gelled, sterile 4.25-cm black filter paper discs (Ahlstrom Filtration, no. 8613–0425) were placed on the media surface. Early-stage embryos, grown in medium 16, were settled for 20 min, and spent medium above the settled cells was removed. Settled cells were gently stirred to mix settling layers, and 1-ml aliquots were pipetted and spread onto the black filter paper. If cell suspensions were clumped, cells were manually spread over the filter paper surface with sterile forceps. 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) was substituted for the filter paper. Four to five replications for each treatment-genotype combination were plated. The 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, observations on embryo production 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 of either germination medium 55 (1/2-strength Murashige and Skoog (1962) 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 100×20-mm petri plates. The 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:soil). Tall magenta boxes were filled with 45 g of moistened soil and autoclaved. The boxes were opened and allowed to air out overnight in a sterile laminar airflow hood to reduce the levels of potential toxic material produced by heating the peat moss contained in the soil mix. The 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 2 weeks thereafter, 2.0 ml of 1/4-strength Schenk and Hildebrant (1972) salt solution was added as fertilizer. The containers were incubated under 16 h of fluorescent light daily at an intensity of approximately 517 lux. After the seedlings had shown epicotyl growth for 2–4 weeks, they were carefully transferred to leech tubes containing the same soil. The leech tubes were enclosed in nonsterile plastic bags containing a wet polyester pad to moisten the air. Over 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. The bag was then removed, and the seedlings grew normally in the greenhouse environment.

After 1 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, the plants were planted in a field plot at the Union Camp Ogeechee Forest in Tattnall County, Georgia. The spacing between seedlings was 3.05×1.83 m. Plant survival and height were tracked for the next two growing seasons.

Cryogenic storage and culture retrieval

The method of Kartha et al. (1988) was used. Embryogenic suspension cultures were grown in medium 16. Five to seven days after subculture, a 30-ml aliquot of settled cells was added to 120 ml of medium 16 supplemented with 0.2 *M* sorbitol and grown for 24 h. Cells were settled and subcultured to medium 16 supplemented with 0.4 *M* sorbitol for another 24 h. The cells were again settled, 53 ml of liquid was removed, the remaining 97 ml was placed into a 1-liter flask, and 1.2 ml of filter-sterilized dimethyl sulfoxide (DMSO) was added slowly. The mixture was cooled on ice for 15 min before an additional 1.2 ml of DMSO was added. Fifteen minutes later, a final aliquot of 1.1 ml of DMSO was added to bring the cryoprotectant concentration to 5%. A 1.8 ml suspension/cryoprotectant mixture was pipetted into 2 ml Nalgene cryogenic vials and placed into a programmable freezer. The vials were first cooled to −35°C at a rate of 0.33°C per min, then transferred to a cryobox and submerged in a liquid nitrogen storage chamber for long-term storage.

To retrieve the cultures, we first removed the vials from the liquid N_2 and thawed them in a 37°C water bath for 5 min. We then opened the vials, flamed them to sterilize the vial lip, and poured the contents onto a sterile, black filter disc (4.25 cm) placed on 20 ml semisolid medium 16+2.5 g/l Gelrite in a petri dish (100×15 mm). After 1 h, the filter paper overlain with cells was moved to a plate of fresh medium. Eighteen hours later, the cells were again transferred to gelled medium 16. The plates were in kept in the dark.

Evaluations of somatic embryo quality

Somatic embryos from medium 240 appeared to be morphologically similar to zygotic embryos at stage 8–9.1 (Pullman and Webb 1994). To confirm our observations, we compared somatic and zygotic embryos 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 Corporation [now International Paper (IPST)] at Belville, Georgia, and BC-1 (S4PT6) from Boise Cascade Corporation at Lake Charles, Louisiana. The cones were shipped on ice to IPST, received within 24–48 h, 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, and then processed to obtain data on 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. The 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 these 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, seeds were cracked using a hemostat, pried open with a scalpel, and the integument, nucellus, and female gametophyte (megagametophyte) were removed. About 50–120 embryos were tested per developmental stage with a maximum of ten 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). Polymerase chain reaction (PCR) analyses were performed with plasmid DNA to amplify the insert region. Five micrograms of PCR product in 15 µl was 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 NaCl-$NaH_2PO_4.H_2O$-Na_2EDTA buffer (20× SSPE containing 5% gel loading dye) was added to the denatured DNAs, which were then blotted onto Hybond N+ membranes (Amersham, UK) as arrays using a VP 386 pin blotter (V&P Scientific, San Diego, Calif.). Each DNA was dot-blotted four times as a quadrate on the membrane. The dots were approximately 1.2 mm in diameter and each of them contained about 30 ng DNA. The DNA was then cross-linked to the membrane at 120,000 mJ/cm^2 in a CL-1000 UV-linker (Upland, Calif.) prehybridized with hybridization buffer (0.5 *M* Na-phosphate, pH 7.2, 5% sodium dodecyl sulfate, 10 m*M* EDTA) at 65°C for 30 min. Probes were made by Advantage PCR (Clontech, Palo Alto, Calif.) using first-strand cDNAs generated by SMART cDNA synthesis kit (Clontech) as templates. The 50 µl PCR reaction mix contained 1× PCR buffer, 5 µl dATP+dGTP+dTTP (5 m*M* each), 1 µl T21VN primer (10 µ*M*), 1 µl SCSP oligo (5'-ctcttaattaagtacgcggg-3', 10 µ*M*), 5 µl template (first-strand cDNA), 1 µl KlenTag enzyme mix, and 5 µl [^{32}P]-dCTP (Amersham). The cycle conditions were one cycle at 94°C for 2 min; 15 cycles of 95°C for 15 s, 52°C for 30 s, 68°C for 6 min. The PCR products were purified using a NICK column (Pharmacia, Piscataway, N.J.) according to the manufacturer's instructions. Hybridization was performed in 3 ml of hybridization buffer in a hybridization oven at 65°C overnight. The membrane was then washed three times with 0.1× hybridization buffer at room temperature for 10 min and once at 42°C for 30 min. The autoradiograph images were scanned and digitized by 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 (Fig. 1). Stage 9 zygotic embryos were also categorized by the week they were collected; for example, 9.1 (stage 9, week 1), 9.2 (stage 9, week 2). 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 classifying both zygotic and somatic embryos to compare early-, mid-, and late-stage development. This system was useful in helping us 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 1/2 P6 modified (Gupta and Pullman 1990). The 1/2 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 2). Microscopic examination of the cultures showed differing morphologies, varying from mostly single cells or clumps to well-formed somatic embryos that resembled early zygotic embryos at stages 1–3 (Figs. 1, 2A). 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 viable, suggesting that establishment in liquid medium 16 shortly after initiation may prevent or delay

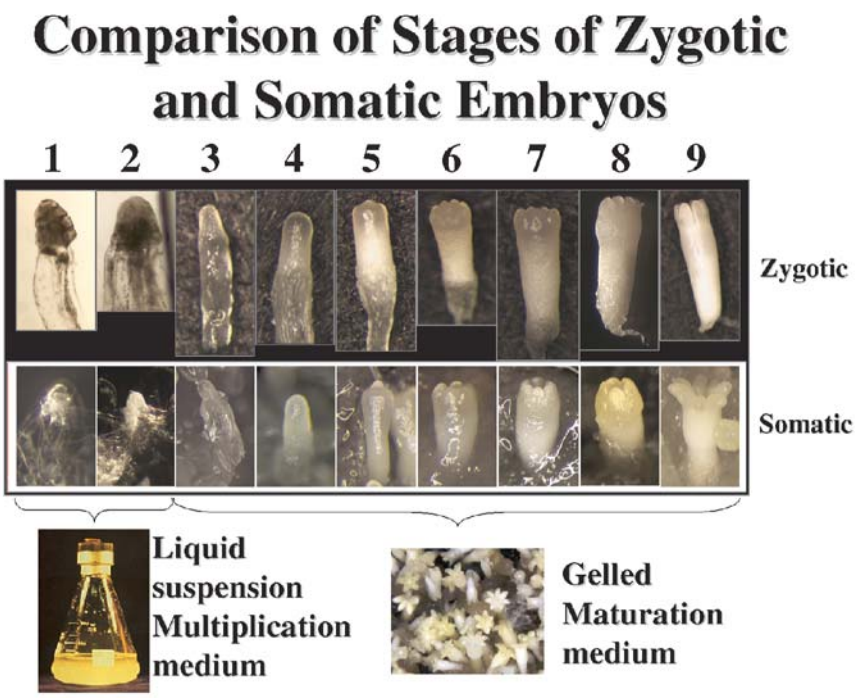

Fig. 1 Comparison of stages of zygotic embryos during natural development and somatic embryos developed on medium 240 over a 3-month period

Table 2 Loblolly pine culture performance: starting liquid cultures, weekly growth rate as settled cell volumes, maximum stage in liquid culture (medium 16), cotyledonary embryo yield in maturation medium 240, and cryogenic survival

Culture no.	Seed origin[a]	Settled cell volume growth rate (5 week average)	Maximum weekly stage rating over 5 weeks	Cotyledonary embryos/ml plated cells; repeated platings	Germination (epicotyl and root growth) on medium 55	Cryogenic Survival
245	UC5-1036	2.4	2	2.6, 15.8	Not tested	3/3
246	UC5-1036	1.6	2	0, 0	–	1/3
247	UC5-1036	1.8	2	1.2, 2.5, 5	Not tested	3/3
248	BC-3	No start[b]	–	–	–	–
249	WV-F2	3.2	2.5	9.6, 0	Not tested	3/3
250	WV-F2	3.6	2	0, 0	–	2/3
251	WV-F2	No start[c]	–	0, 23	–	3/3
252	BC-9	2.7	3	7.8, 50+, 25	Not tested	2/3
253	UC10-33	4.6	2	0.8	Not tested	3/3
254	UC10-33	2	2	0, 11	Not tested	3/3
255	BC-9	1.8	2	18.6, 12.3, 15	0/70 (0%)	1/3
256	WV-F2	1.3	2	7.4	Not tested	0/3
257	UC10-33	3.2	3	8.4, 10.5	1/70 (1%)	3/3
258	UC10-5	No start[b]	–	–	–	–
259	BC-9	2.1	3	39.8, 23.6, 41.6	0/500 (0%)	3/3
260	BC-3	1.5	2	8.0, 21.2	0/20 (0%)	2/3
261	UC5-1036	1.4	3	40.8, 33.8	16/349 (5%)	3/3
262	WV-I2	1.9	1	0	Not tested	3/3
263	UC10-5	No start[b]	–	–	–	–
264	WV-F2	No start[b]	–	–	–	–
265	UC5-1036	No start[c]	–	–	–	–
266	UC10-33	2.3	3	10.8, 1.3	33/100 (33%)	3/3
267	UC10-33	2.4	2	24, 4.5	20/151 (13%)	3/3
268	UC5-1036	4.7	1.5	1.6, 3.7	0/20 (0%)	3/3
269	BC-2	No start[b]	–	–	–	–

[a] Origin of seed materials used for culture initiation: UC, Union Camp Corporation; BC, Boise Cascade Corporation; WV, Westvaco Company

[b] Embryogenic tissue was not available for a second attempt to start liquid cultures

[c] During a second try, liquid cultures were successfully started

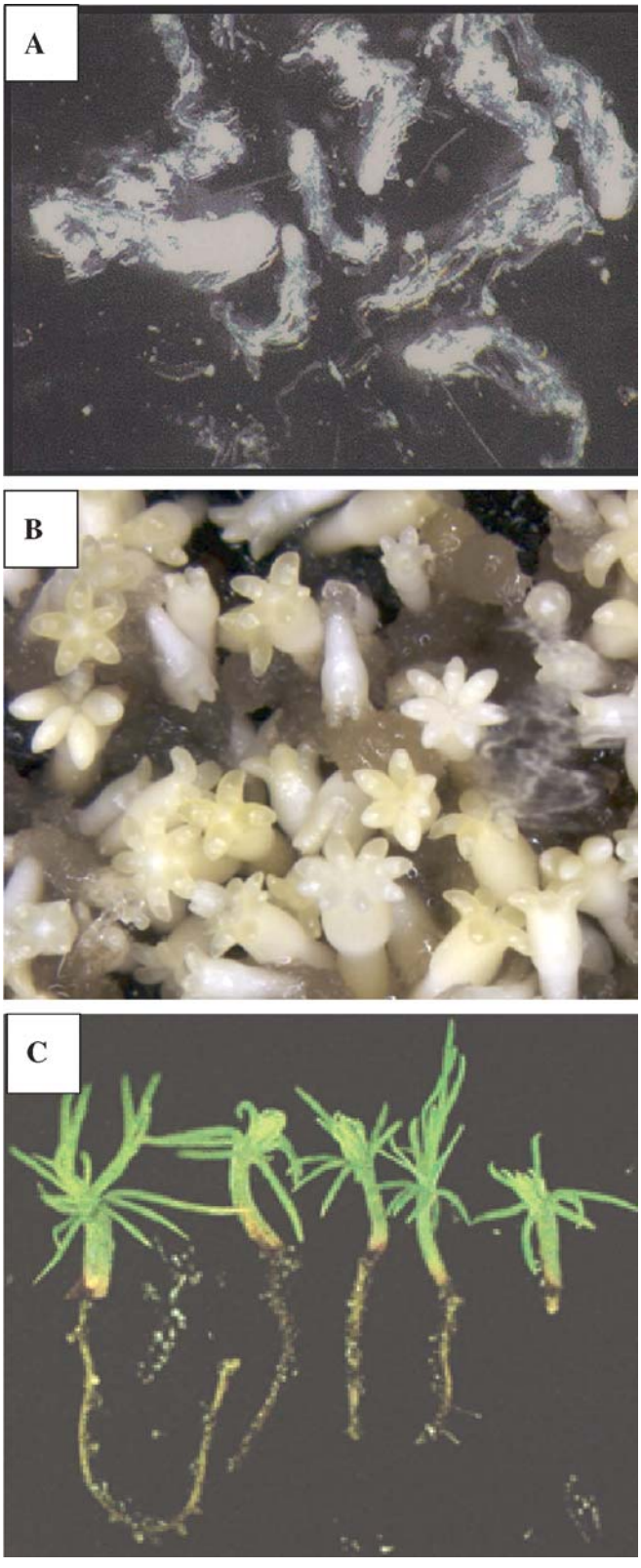

Fig. 2A–C Loblolly pine somatic embryos. **A** Grown in medium 16; stage 2 somatic embryos are visible. **B** Cotyledonary embryos grown on medium 240; stage 8–9.1 somatic embryos are visible. **C** Stage 8–9.1 somatic embryos germinated on medium 397, showing root and shoot growth

the loss of a culture. Average weekly multiplication rates over 5 weeks ranged from 1.5- to 4.7-fold per week. Each week, several drops of embryo suspensions from each culture were examined and scored for the most advanced embryo stages present; the stages present varied from stage 1 to stage 3. A maximum stage rating of at least two was seen in 16/18 (89%) 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. Nineteen of the liquid-grown cultures were placed in cryogenic storage.

The observation that only liquid cultures with organized early-stage embryos (stages 1.5–2) produced 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 the placement of unorganized cultures into the next developmental step of the protocol. Other researchers have also reported stage-related responses, such as for 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 20 ml of maturation medium in a 100×15-mm petri plate. Transfers to new medium were carried out every 2 weeks for a total of four transfers. At the end of 3 months, the cultures were observed for presence of cotyledonary embryos. Of 53 genotypes tested, only two produced cotyledonary embryos. The few cotyledonary embryos produced (two and eight) were of low quality, short, barrel-shaped in appearance, and embedded in tissue. These embryos were not able to germinate when placed on medium 55.

Polyethylene glycol replacement of maltose

The results from screening 53 genotypes on medium 24 were promising with respect to embryo production but required significant improvements in embryo yield and quality. As polyethylene glycol (PEG) had been reported to improve embryo yield and quality in both white spruce (Attree et al. 1991) and Douglas fir (Gupta et al. 1993), we began to test its effect by substituting 2% and 4% of the maltose with 7.5% and 13% PEG, respectively, while

Table 3 Medium osmolality and number of normal and total cotyledonary somatic embryos produced per milliliter of settled cells for maturation media varying in basal salts, maltose, and polyethylene glycol content (*SE* standard error)

Medium	Osmolality (mmol/kg)	NCE[a] and TCE[a]/ml cells (±SE)					
		Genotype 31		Genotype 41		Genotype 195	
		NCE	TCE	NCE	TCE	NCE	TCE
24	257	0.3 (±0.3)	0.5 (±0.5)	0	1.0 (±0)	2.5 (±1.6)	8.0 (±3.0)
225	253	1.0 (±1.0)	1.0 (±1.0)	0	0.5 (±0.3)	151 (±11.8)	161 (±13.0)
239	246	0	0.7 (±0.3)	0	1.5 (±0.6)	6.0 (±2.3)	41.8 (±3.9)
240	259	30.8 (±6.3)	46.0 (±6.3)	0	6.8 (±1.8)	225.0 (±23.6)	230.8 (±24.3)

[a] NCE and TCE, Normal and total cotyledonary embryos, respectively

maintaining osmolality at approximately 240 mmol/kg. Five replications for each of three genotypes, grown in medium 16, were plated onto maturation media 24, 226 [maltose reduced to 4%, and 7.5% PEG (MW 8,000) substituted as an osmoticant], or 225 (maltose reduced to 2% and 13% PEG substituted as an osmoticant).

An effort was made to measure the osmolality of the maturation media under test and to compare media of 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 versus percentage PEG (8,000) (%PEG) added to basal medium containing 2% maltose with the pH adjusted to 5.8. A highly significant curvilinear relationship (r^2=0.964) was obtained for %PEG versus medium osmolality (mmol/kg). By moving along the curve to the osmolality of the basal without PEG, we were able to predict the osmolality increase that a defined addition of PEG would cause. 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 was clearly beneficial to 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 (Fig. 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, the embryos developed further, emerging from the callus and increasing yield slightly. The three media tested had similar osmolality measurements, ranging from 227 mmol/kg to 233 mmol/kg. When tested for germination on medium 55 (Table 1), none of the embryos were able to produce a root and shoot.

Based on the results of 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 the 29 cultures plated on medium 225 (13% PEG, 2% maltose), one culture was contaminated, and 5 of the remaining 28 produced cotyledonary embryos. Four of the five cultures produced fewer than five cotyledonary embryos per milliliter settled cells.

In a third experiment, four different molecular weight polyethylene glycol types were tested for cotyledonary embryo production. The PEG molecular weight and percentage added to the 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 for four loblolly pine genotypes. The four PEG molecular-weight 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 milliliter 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 8,000 and were significantly greater by the Duncan Multiple Range test (P=0.5), than on all of the other PEG molecular weights.

MSG salts versus 1/2 P6m salts and maltose versus maltose/PEG

Maturation medium 24 is based on MSG salts. However, as embryos showed good growth in the initiation and maintenance steps on media containing modified 1/2 P6 salts and casamino acids, we decided to compare 1/2 P6m salts with MSG salts in maturation media in combination with 0 or 13% PEG, with 6% or 2% maltose, respectively (media 24, 225, 239, 240; Table 1). Four genotypes were tested with four replications per genotype and treatment.

Medium osmolality ranged from 246 mmol/kg to 259 mmol/kg for the test media (Table 3). One genotype did not form cotyledonary embryos on any test medium, while the remaining three genotypes showed high yields of cotyledonary embryos in medium 240 (1/2 P6m salts, 2% maltose, 13% PEG; Table 3). Both embryo yield and quality improved with the combination of modified 1/2 P6 salts and PEG. In addition to higher yields, cotyledonary embryos developed faster, had a smoother surface (less callus), and were shaped more like zygotic embryos, showing distinct root, hypocotyl, and cotyledon regions (Fig. 2B). Statistical analysis of the data from the three cotyledonary embryo-producing genotypes by the Duncan Multiple Range test showed no significant differences in normal or total cotyledonary embryos when MSG salts were compared to 1/2 P6m salts with 6% maltose. However, in the presence of 13% PEG, 1/2 P6m produced

greater yields of normal and total embryos compared to those from MSG (P=0.05). PEG (13%) resulted in a statistically significant increase in cotyledonary embryos (P=0.05) when tested in MSG or 1/2 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

Of 25 new cultures grown in medium 16 for at least five subcultures, 20 were plated onto medium 240 (Table 2). Embryos from most of these genotypes became visible to the naked eye during the second and third subcultures on maturation medium. Fourteen of the genotypes (56%) produced cotyledonary somatic embryos morphologically similar to zygotic stages 6–9.1. Across these 14 genotypes, yields on maturation medium 240 averaged 10.6 cotyledonary embryos per milliliter settled cells. Genotypes with stages 7–9.1 embryos and with suitable numbers of embryos were tested for germination on medium 55. Four out of the eight genotypes tested produced germinants with germination ranging from 1% to 33%.

Maturation improvement

The combination of 2% maltose, 13% PEG 8,000, and 1/2 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 the water potential of developing loblolly pine ovules. As embryos developed from stage 2 to stages 4–6, ovule water potential dropped 100–200 mmol/kg to 200–300 mmol/kg. Sucrose has been reported to breakdown in conifer maturation medium into glucose and fructose, thereby causing medium osmolality to increase (Iraqui and Tremblay 2001). This hydrolysis of the sucrose in the medium has been attributed to cell-wall invertase (Yu et al. 2000). The use of sucrose during stages 4–6 causes the osmolality to rise while maltose allows osmotic levels to remain static or slowly decline (unpublished 1997 data). We speculate that maltose is a superior carbohydrate/osmoticant because it facilitates the creation of a more natural osmotic environment during the critical developmental stages 4–6.

Li et al. (1997, 1998) also used the results of Uddin (1993), Pullman and Webb (1994), Gupta and Pullman (1990), 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 a maltose/PEG combination improved embryo maturation for two loblolly pine genotypes. However, germination was not assessed. 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 (3,500). In our work, the combination of maltose PEG (8,000) and Gelrite (2.5 g/l) produced a superior somatic embryo. Patent coverage (Pullman and Gupta 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.

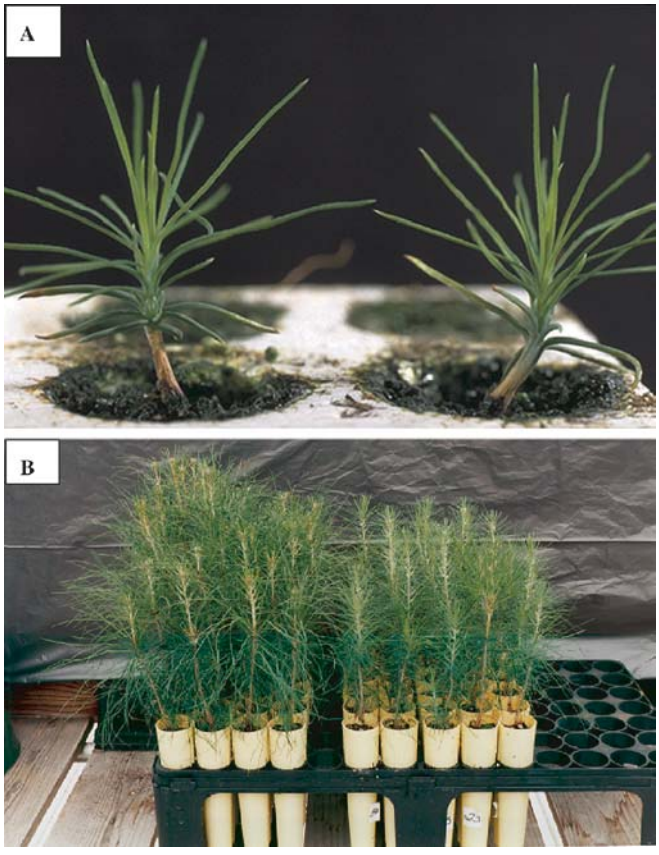

Fig. 3A, B Somatic seedlings of loblolly pine. **A** Acclimated somatic seedling growing in the greenhouse, **B** greenhouse-grown somatic seedlings during a spring flush of new growth

Germination of somatic embryos

Observations of germination tests with ten different genotypes showed that somatic embryos responded during the first 4–5 weeks by initially expanding the hypocotyl followed by expansion of the cotyledons. During the next 4–5 weeks, apical primordia appeared that slowly expanded into needle growth. After approximately 2 months, root growth began (Fig. 2C). Both root and shoot growth continued slowly. While this sequence of germination is slow and awkward compared that of 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 were 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. Initially, germinants appeared to spend several weeks in an acclimation period. They were a dull, dusty-green color with no apparent growth of the epicotyl or hypocotyl. 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, but after 1–2 weeks began growing again as bright green needles that spread out. At this stage, the seedlings were gently transferred to leech tubes in nonsterile transparent plastic bags that were gradually opened to the culture room and later to the greenhouse environment. Across all germinants, 30/59 (51%) survived conversion and acclimation and continued growth in the greenhouse (Fig. 3A, B). Higher survival occurred with improved germinant quality.

In October of the planting year, all of the 35 field-grown trees from somatic embryos had survived and averaged 1.9 feet in height. Trees 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. A *t*-test comparison of these two populations showed a statistically significant difference at $P=0.05$. It is speculated that the differences in height may be due to a slow start in the first growing season due to somatic seedling residency in our greenhouse for 1 year.

Cryogenic storage and culture retrieval

Growth first appeared after 3 weeks to 2 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 the tissue was further maintained in medium 16 or on gelled medium 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 showed an increase in dry weight and a decreased percentage of water content as embryo stage advanced. Somatic embryos from genotypes with the most advanced development, resulting from 11–13 weeks of growth on medium 240, ranged from 0.43 mg to 0.98 dry weight per embryo. Full-term zygotic embryos at stage 9.8 ranged in dry weight from 1.4 mg to 1.6 mg per embryo. Somatic embryo morphology and dry weights were most similar to those of the zygotic embryos at stages 8–9.1.

Table 4 Zygotic and somatic embryo dry weights and water content

Zygotic embryo stage	Tree S4PT6			Tree UC5-1036		
	Embryo dry weight (mg)	Embryo dry weight standard error (mg)	Ovule water content (%)	Embryo dry weight (mg)	Embryo dry weight standard error (mg)	Ovule water content (%)
6	0.04	0.01	62	0.07	0.01	62
7	0.08	0.01	56	0.09	0.01	45
8	0.17	0.03	48	0.28	0.04	47
9.1	0.50	0.03	39	0.91	0.07	36
9.2	0.92	0.02	34	1.05	0.12	34
9.8	1.44	0.19	23	1.63	0.07	27
Somatic embryo genotype	Somatic embryo dry weight (mg)	Somatic embryo dry weight standard error (%)	Somatic embryo water content (%)	Somatic embryo water content standard error (%)		
195	0.52	0.10	82.2	1.4		
260	0.94	0.12	80.4	0.7		
266	0.78	0.12	80.9	0.3		
275	0.43	0.05	76.7	0.8		
314	0.98	0.11	79.8	0.8		
333	0.71	0.03	79.6	0.7		

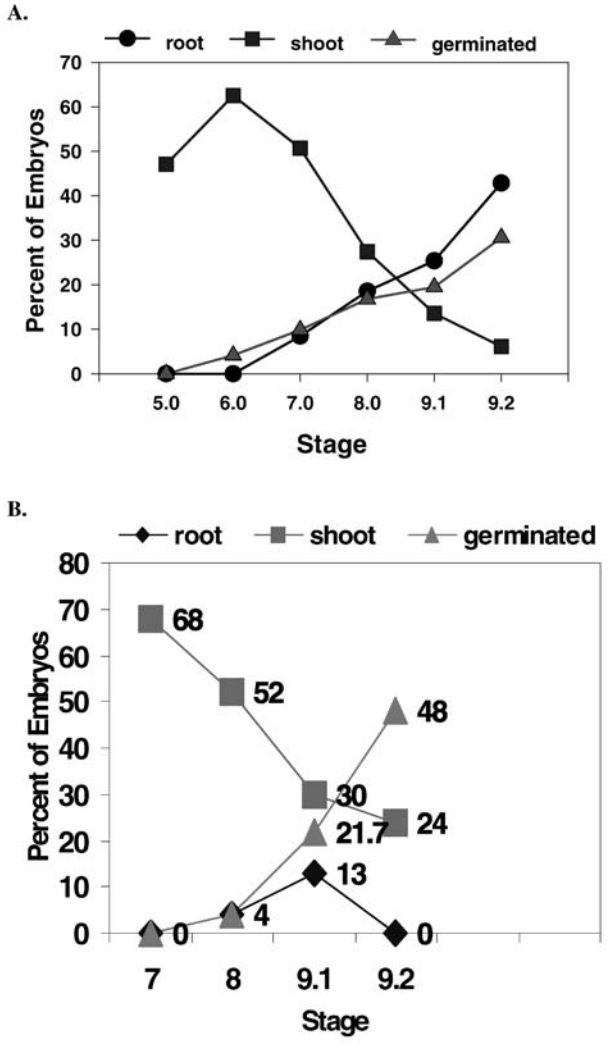

Fig. 4A, B Germination of zygotic loblolly pine embryos isolated at various stages of development. **A** Germination of UC5-1036 stage 5–9.2 embryos obtained in 1997, **B** germination of S4PT6 stage 7–9.2 embryos obtained in 1998

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, then shoot emergence and then, much later, if at all, the appearance of the root. 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 became competent to germinate. It also allowed us to compare germination for zygotic and somatic embryos, thereby providing estimates of the somatic embryo developmental stage. The results indicate that our somatic embryos developed to stages 7–8, suggesting that early somatic embryo development, stages 1–6, occurred normally and that our block was in the later stages of embryo development on maturation medium.

Most embryos survived the sterilization and isolation process, with 80% and 100% survival at stages 5 and 9.2, respectively, 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. At stage 5, 60% of the zygotic embryos produced 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 the germination tests, whereas those in 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 roots, suggesting that functional root development began 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 the stage progressed past stage 7. Shoot production, root production, and germination (both shoot and root production) for the UC5-1036 and S4PT6 cone collections at stages 5–9.2 are shown in Fig. 4. Both embryo collections started germinating at stages 7–8, and the germination percentage increased with stage. This observation suggests that zygotic embryos developed the capability to germinate at stages 7–8. In comparison, zygotic embryos at stages 7–8 and our most advanced somatic embryos showed a similar germination performance in both sequence and percentage germination.

A functional transition appeared to occur in zygotic embryos between stages 8 and 9.1 with respect to germination. At stages 9.1 and 9.2, there were more embryos that only had roots than ones with only shoots or that germinated. This result 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. In embryos at stage 9.1 and beyond, the root meristem was probably activated first upon germination. This is the natural sequence for mature zygotic embryo germination from seed. Thus, before development had reached stage 7, neither zygotic nor somatic embryos had the capacity to germinate in the root-shoot sequence observed for fully mature zygotic embryos. In maturing embryos, a developmental transition occurred in the ability of the root or shoot to form first during zygotic embryo germination.

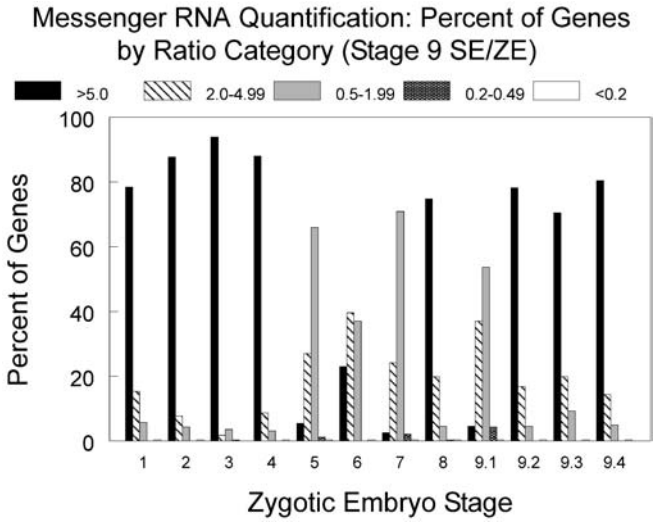

Fig. 5 Messenger RNA quantification ratios for stage 9 somatic embryos/zygotic embryos at varying stages. Resulting ratios for gene activity are categorized, and the percentage of the gene collection is presented by category

Zygotic and somatic embryo gene expression

Somatic embryos appeared to develop normally through stages 1 to 8 and then stopped further growth. At the end of development on maturation medium 240, somatic embryos were often similar to zygotic embryos at stage 8, having a similar morphology and dry weights between stages 8 and 9.1 and a comparable germination sequence to that of 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 stages 7–9.1 they should show a gene expression activity similar 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 the expression activity for that gene in zygotic embryos at stages 1 through 9.4. A ratio of 1 indicates that the mRNA level for a given gene is identical in both types of embryo; a ratio greater than 1 indicates that the mRNA level is higher in the somatic embryo than in the zygotic embryo to which it is being compared; a ratio less than 1 indicates higher zygotic embryo expression. The resulting ratios of gene activity were categorized by degree of difference – greater than 5.0, 2.0–4.99, 0.5–1.99, 0.2–0.49, and less than 0.2 – and the percentage of genes falling into each category was calculated and plotted (Fig. 5). Gene expression of stage 9.1 somatic embryos was found to be 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 to stage 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, which is consistent with our previous conclusion that our most advanced somatic embryos are similar to those in 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 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 medium 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 SE process to become commercial. With the successful storage of cultures in liquid nitrogen shortly after initiation, we hope to prevent culture decline over time, decrease the labor necessary to maintain cultures, and have available a bank of cultures with known histories of performance. Research could then focus on the step or embryo stage where improvement is needed.

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