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Original article

Somatic embryogenesis in loblolly pine (*Pinus taeda* L.): improving culture initiation rates

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Abstract – Loblolly pine (*Pinus taeda* L.) is one of the most important commercial trees in the U.S. To be successful for commercial use, somatic embryogenesis technology must work with a variety of genetically diverse seeds. Initiation rates of loblolly pine were improved through a combination of modified 1/2 P6 salts, activated carbon at 50–100 mgL^{-1}, Cu and Zn added to compensate for adsorption by activated carbon, 1.5% maltose, 2% myo-inositol, (to raise osmotic level partially simulating the ovule environment), 500 mg L^{-1} case amino acids, 450 mg L^{-1} glutamine, 2 mg L^{-1} NAA, 0.45 mg L^{-1} BAP, 0.43 mg L^{-1} kinetin, and 1.6–2 g L^{-1} Gelrite. Across 10 open-pollinated families, initiation rates ranged from 3–33%, averaging 16%.

Loblolly pine / somatic embryogenesis / initiation / conifer / *Pinus taeda*

Résumé – Embryogenèse somatique de *Pinus taeda* L. : amélioration du taux d'initiation des cultures. *Pinus taeda* L. est l'une des plus importantes espèces commerciales aux États-Unis d'Amérique. Pour être exploitable à un niveau commercial, l'embryogenèse somatique doit s'appliquer sur une grande variété de graines génétiquement diverses. Les taux d'initiation de cultures embryogènes de *Pinus taeda* ont été améliorés grâce à l'association des éléments suivants : sels du milieu P6 dilué de moitié, charbon actif à la dose de 50 à 100 mg L^{-1} (avec adjonction de Cu et Zn pour compenser leur adsorption par le charbon actif), 1,5 % de maltose, 2 % de myo-inositol (pour augmenter la pression osmotique, simulant partiellement le développement de l'ovule), 500 mg L^{-1} d'acides aminés provenant d'hydrolyse acide de caséine, 450 mg L^{-1} de glutamine, 2 mg L^{-1} d'acide naphtalène acétique, 0,45 mg L^{-1} de benzylaminopurine, 0,43 mg L^{-1} de kinétine et 1,6 à 2 mg L^{-1} de Gelrite. Dans ces conditions, les taux d'initiation varient pour les 10 descendances maternelles étudiées de 3 à 33 %, avec une moyenne de 16 %.

***Pinus taeda* / embryogenèse somatique / initiation / conifère**

1. INTRODUCTION

Conifer somatic embryogenesis (SE) has been demonstrated for many genera [7, 14, 20, 21]. SE proceeds through initiation, multiplication, maturation, and germination. A cryogenic storage step may be added when storage of embryogenic cultures is desired. The first report of SE in loblolly pine (LP) (*Pinus taeda* L.) occurred in 1987 [8]. Since then several reports have focused on LP along with abundant patent activity [3, 18]. Factors currently limiting commercialization of SE for LP include low initiation rates (many desirable genotypes are recalcitrant), low culture survival, culture decline causing low or no embryo production, and the inability of somatic embryos to fully mature resulting in low germination and slow initial growth of somatic seedlings.

Reports on initiation of LP embryogenic tissue indicate initiation frequencies of 1–5% [1, 3, 8, 12, 13]. Several patents contain methods for improved initiation frequencies for LP [2, 10, 11]. These low levels have provided a block for the scientific and commercial use of SE to multiply valuable LP genotypes. To capture the gains of long-term LP breeding programs, clonal propagation methods must work on a wide range of genotypes.

Activated carbon (AC) improved initiation in radiata pine and embryo development in Douglas-fir [16, 19]. Since AC may adsorb 95–99% of the plant growth regulators (PGR)

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present in tissue culture media [5, 6, 16], we began to test initiation media with greatly increased levels of PGR combined with 2.5 g L^{-1} AC. Statistically significant increases in ovule extrusion occurred when AC was added to the initiation medium; however, only a few initiations resulted. Toering (1995) [24] tracked the availability of 2,4-dichlorophenoxyacetic acid (2,4-D) in initiation media using radiolabeled 2,4-D. Media with 2.5 g L^{-1} AC and 220 mg L^{-1} 2,4-D contained 12–17 mg L^{-1} available 2,4–D during much of the initiation period. These findings suggested two approaches to improve initiation media: (1) lower 2,4-D from 220 to 110 mg L^{-1} in the presence of 2.5 g L^{-1} AC; or (2) greatly reduce AC levels and combine with standard or slightly raised PGR levels similar to levels used in media without AC.

When somatic embryos from suspension cultures were placed on initiation media without AC they grew well; however, when media contained 2.5 g L^{-1} AC growth was reduced. This observation suggested that AC adsorbs a required media component or adds a toxic component to the medium. With this working hypothesis, media mineral components +/– AC were analyzed [17, 25] and AC at 2.5 g L^{-1} was found to reduce Cu 90% and Zn 50%.

The focus of the research in this report was the development of a somatic embryogenesis (SE) initiation system that would work across a diversity of genetic material of *Pinus taeda*. Measurements of ovule water potential during early embryo growth and availability of ions and PGR in tissue culture media containing AC provided clues for SE initiation improvements. Simulated initiation tests using single somatic embryos as explants were used to test potential medium improvements and zygotic embryo initiation tests verified improvements.

2. MATERIALS AND METHODS

2.1. Plant materials

2.1.1. Zygotic embryos

Loblolly pine cones were collected weekly in early to mid-July 1993–1998 from open-pollinated trees in clonal seed orchards, shipped on ice, and received within 24–48 hours. Collections also occurred in mid-January by Westvaco Corp./Rigesa, Celulose from breeding orchards near Canhinhas, Santa Catarina, Brazil. Cones were stored at 4–5 °C for 1–9 weeks.

Cones containing seeds with embryos mostly at stages 2–4 [18] were used for initiation tests. Cones were opened and seeds separated from the ovuliferous scales. Seeds were washed in running cold tap water for 10 min, agitated for 10 min in 10% liquinox (detergent) containing 2 mL Tween 20 L^{-1}, rinsed for 30 min with running tap water, agitated for 10 min aseptically in 20% (v/v) hydrogen peroxide, and rinsed five times with sterile deionized water. Dissection: seeds were cracked using a hemostat, pried open with the aid of forceps and a scalpel, and the seed coat, integument and nucellus were removed from the ovule (megagametophyte).

2.1.2. Somatic embryos

Somatic embryos were also used as explants for experiments. Cultures of somatic embryos were multiplied in liquid medium 16 [18]. Single stage 2 embryos were isolated with forceps from suspension culture and placed on test initiation medium, grown for 4–7 weeks, and measured for resulting embryogenic tissue diameters. Thirty or forty single somatic embryos were evaluated on test media arranged in three or four replicates of ten embryos each. Single somatic embryo explants provided a measurement of a medium's potential to support the last phase of initiation and multiplication of the somatic embryos into an embryogenic culture mass.

2.2. Media and Culture Conditions

Medium most often consisted of modified 1/2 P6 Salts [9], a modification of P6 from Teasdale et al. 1986 [22]. Acid-washed tissue culture tested AC (Sigma, C9157) was used in media containing AC. The pH of medium was adjusted with KOH or HCl after the addition of all ingredients except gelling agent or filter-sterilized materials. 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 approximately 55 °C. Maltose was used as a carbon source and medium was gelled with 1.6–4 g L^{-1} gellan gum. Two % myo-inositol was included in media to raise osmolality from approximately 160 to 225 mmol kg^{-1} based on measured water potential of developing LP ovules [15]. Explants were cultured on 2 mL of medium contained in individual wells of Costar #3526 Well Culture Cluster Plates. Petri dishes and well plates were wrapped in two layers of Parafilm and incubated at 23–25 °C in the dark.

2.3. Extrusion and initiation success and statistical analysis

Initiation occurs in three steps. Within 1–4 weeks extrusion occurs when one or more zygotic embryos push out of the megagametophyte micropylar end and become visible protruding from the gametophyte or on the medium. At 5–7 weeks somatic embryos begin to form on the zygotic embryos. Somatic embryos then continue the multiplication process to form a mass of embryogenic tissue. These phases can be evaluated as % extrusion, % explants forming three or more somatic embryos (visible through a dissecting microscope), and % explants achieving a target mass or size. Percent extrusion and explants with three or more somatic embryos visible were routinely evaluated 9–10 weeks after placement of ovules on media. Treatments were arranged in a completely randomized design. Data were analyzed by analysis of variance and significant differences between treatment means were determined by the Duncan Multiple Range Test at the 5% level of significance.

2.4. Adjustment of copper and zinc compensating for AC adsorption

Medium 288 (*table I*) was prepared and Cu sulfate adjusted to 0.125 (medium 288), 0.25, 0.375, 0.5, 1.0, and 2.5 mg L^{-1}. Thirty somatic embryos from each of three genotypes were placed on test medium as described earlier. After seven weeks tissue diameters were measured.

Table I. Media components for experiment varying activated carbon.

	Media (mg L^{-1})								
Components	201	288	504	505	506	507	508	509	539
NH_4NO_3	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0
KNO_3	909.9	909.9	909.9	909.9	909.9	909.9	909.9	909.9	909.9
KH_2PO_4	136.1	136.1	136.1	136.1	136.1	136.1	136.1	136.1	136.1
$Ca(NO_3)_2$•$4H_2O$	236.2	236.2	236.2	236.2	236.2	236.2	236.2	236.2	236.2
$MgSO_4$•$7H_2O$	246.5	246.5	246.5	246.5	246.5	246.5	246.5	246.5	246.5
$Mg(NO_3)_2$•$6H_2O$	256.5	256.5	256.5	256.5	256.5	256.5	256.5	256.5	256.5
$MgCl_2$•$6H_2O$	101.7	101.7	101.7	101.7	101.7	101.7	101.7	101.7	101.7
KI	4.15	8.3	4.15	4.15	4.15	4.15	4.15	4.15	4.15
H_3BO_3	15.5	31	15.5	15.5	15.5	15.5	15.5	15.5	15.5
$MnSO_4$•H_2O	10.5	21	10.5	10.5	10.5	10.5	10.5	10.5	10.5
$ZnSO_4$•$7H_2O$	14.4	28.8	14.4	14.688	14.976	15.84	17.28	28.8	14.688
Na_2MoO_4•$2H_2O$	0.125	0.25	0.125	0.125	0.125	0.125	0.125	0.125	0.125
$CuSO_4$•$5H_2O$	0.125	0.25	0.125	0.1725	0.22	0.3625	0.6	2.375	0.1725
$CoCl_2$•$6H_2O$	0.125	0.25	0.125	0.125	0.125	0.125	0.125	0.125	0.125
$NiCl_2$•$6H_2O$	–	0.13	–	–	–	–	–	–	–
$FeSO_4$•$7H_2O$	13.9	13.9	13.9	13.9	13.9	13.9	13.9	13.9	13.9
Na_2EDTA	18.65	18.65	18.65	18.65	18.65	18.65	18.65	18.65	18.65
Maltose	15 000	15 000	15 000	15 000	15 000	15 000	15 000	15 000	15 000
Myo-inositol	20 000	20 000	20 000	20 000	20 000	20 000	20 000	20 000	20 000
Casamino acids	500	500	500	500	500	500	500	500	500
L-Glutamine	450	450	450	450	450	450	450	450	450
Thiamine•HCl	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Pyridoxine•HCl	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Glycine	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
2,4-D	220	220	–	–	–	–	–	110	–
NAA	–	–	2.0	2.0	2.0	2.0	2.0	–	2.0
BAP	90	90	0.45	0.45	0.45	0.45	0.45	45	0.45
Kinetin	86	86	0.43	0.43	0.43	0.43	0.43	43	0.43
Activated charcoal	2500	2500	–	50	100	250	500	2500	50
Gelrite	4000	4000	2 000	2 000	2 000	2 000	2 000	2 000	1600
pH	5.2	5.2	5.7	5.7	5.7	5.7	5.7	5.7	5.7

Inductively coupled plasma (ICP) atomic emission spectroscopy for Zn and graphite furnace atomic adsorption for Cu were performed on six media that were adjusted for initial Cu and Zn levels. Medium 201 (*table I*) was modified by the addition of 0.13 mg L^{-1} $NiCl_2.6H_20$, adjustment of zinc sulfate to 57.6 mg L^{-1}, and adjustment of copper sulfate to 0.25, 1.0, 2.5, 5.0, and 10 mg L^{-1}. Control medium had no AC, copper sulfate and zinc sulfate at 0.125 and 14.4 mg L^{-1}, and 2,4-D, BAP, and Kinetin reduced to 1.1, 0.45, and 0.43 mg L^{-1}, respectively. Three replicates of ten ml per test medium were filtered through a 0.22 µm cellulose acetate membrane to collect liquid for analysis on days 14 and 28 to determine if Cu and Zn were further adsorbed. A water control was included for reference.

2.5. Initiation from ovule explants in media containing low amounts of activated carbon

AC was varied from 0 to 500 mg L^{-1} using PGR levels at 2 mg L^{-1} NAA, 0.45 mg L^{-1} BAP, and 0.43 mg L^{-1} Kinetin, adding extra Cu and Zn, and 2 g L^{-1} gellan gum (*table I*, media 504–508). A control medium containing 2.5 g L^{-1} AC with raised hormones, Cu, and Zn was also included (*table I*, medium 509). Thirty ovules (three replicates of ten) from each of three cone collections and thirty somatic embryo explants were placed on each test medium.

With the positive effects evident for low AC combined with 2 g L^{-1} gellan gum, NAA, and Cu and Zn adjusted for AC adsorption, we next wanted to determine the optimal gellan gum level. Components in medium 505 (*table I*) were held constant and gellan gum was varied from 0–4 g L^{-1}. A control medium with 2 g L^{-1} Gelrite and no AC was also included (medium 504, *table I*). Thirty ovule explants (three replicates of ten) from each of three cone collections were placed on each test medium. Ovules in liquid media were placed on presterilized 3-cm squares of polyester batting covered with a 4.25-cm circle of Ahlstrom Black Filter Paper Grade 8613 contained in 60 × 15 mm Petri plates.

2.6. Initiation rates and culture survival for multiple families

Cultures initiated on media 505 and 539 were maintained on medium 16 [18] with the addition of 2.5 g L^{-1} gellan gum. Embryogenic tissue was subcultured every two weeks. Tissue masses about one

cm in diameter were divided into 2–3 parts at the beginning of each subculture. After six months data were collected on the number of cultures actively growing. Active growth was determined by marking the colony edge at the time of transfer and visually determining at the end of the subculture cycle if the colony size increased.

3. RESULTS

3.1. Adjustment of copper and zinc compensating for AC adsorption

Tests with single somatic embryos grown on initiation medium showed a strong effect of medium Cu content on growth into masses of embryogenic tissue. Colony size increased with increasing Cu concentration in medium supplemented with 2.5 g L^{-1} AC (*figure 1*). Activated carbon in the initiation medium likely adsorbed Cu ions resulting in Cu deficiency. Supplementing medium with extra Cu compensated for the adsorbed Cu removing the deficiency.

In general, free Cu and Zn levels rose when the initial concentrations were increased (*figure 2*). Adding 2.5 mg L^{-1} (20 times the original amount) of $CuSO_4 \cdot 5H_2O$ to charcoal-containing medium duplicated the level of free Cu available in medium without charcoal. When the initial Zn level was quadrupled, it more than compensated for the adsorption by AC. Doubling the $ZnSO_4 \cdot 7H_2O$ to 28.8 mg L^{-1} would probably result in the desired level available in media without AC. There was little difference in Cu or Zn after 14 vs. 28 days.

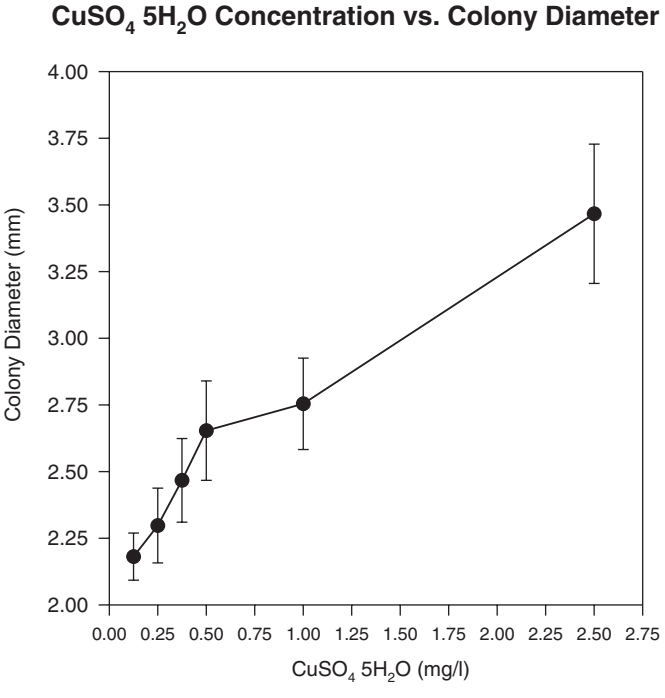

Figure 1. Effect of initial copper sulfate concentration on growth of single somatic embryos into embryogenic tissue masses. Medium contained 2.5 g L^{-1} of activated carbon. Standard error bars are shown for growth at each copper concentration.

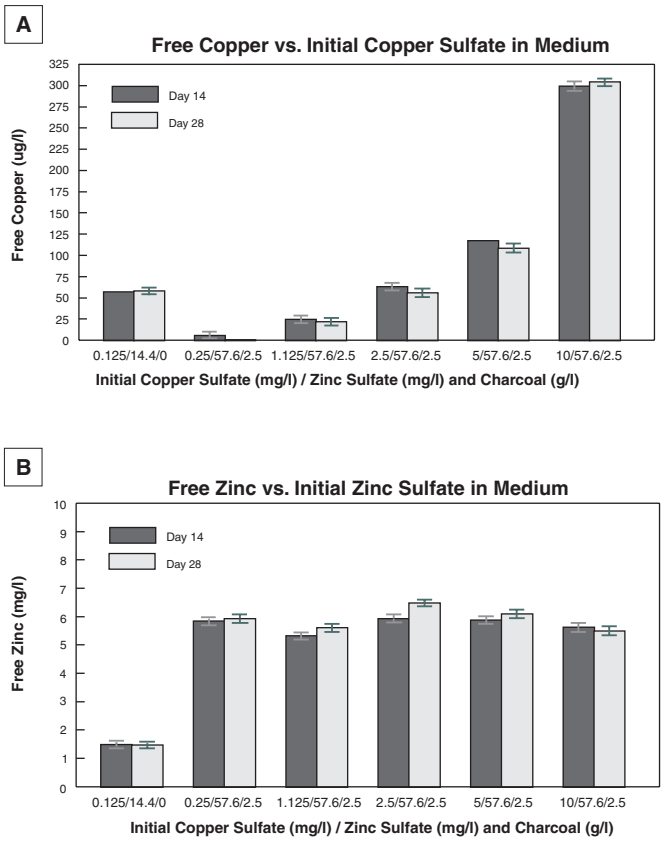

Figure 2. Effect of five copper sulfate/zinc sulfate and two charcoal concentrations on the amount of: (A) free copper (μg L^{-1}) and (B) free zinc (mg L^{-1}) that is available in medium. Standard error bars are shown for each triplicate analyses.

3.2. Initiation from ovule explants in media containing low amounts of activated carbon

Medium containing 50–100 mg L^{-1} AC supported the highest ovule extrusion rates (*table II*). While statistically significant differences did not occur for initiation rates per medium, a trend was suggested for improved extrusion and initiation with lower levels of AC. Medium 505 produced 3–10% SE initiation across three cone collections. Growth of single somatic embryos on test initiation media also supported this observation (*table II*) by being greatest on medium 505 (50 mg L^{-1} AC) and worst on medium 504 (no AC).

Results from varying the gellan gum level in medium 505 are shown in *table III*. Extrusion % and initiation % increased as the gelling agent increased from none to 2 g L^{-1}. Peak extrusion and initiation occurred at 2 g L^{-1} (medium 505). Ovules on liquid medium (0–0.25 g L^{-1} gellan gum) showed the lowest extrusion and initiation rates.

Table II. Extrusion and initiation on media containing low activated carbon levels.

Media	Activated carbon	Extrusion	Seed source and initiation				Embryogenic tissue growth
	mg L^{-1}	Average* (%)	UC 10–33	BC 2	UC 10–5	Average* (%)	Colony diameter* (mm)
504	0	18.9 a	2/30	0/30	0/30	2.2 a	3.9 a
505	50	33.3 b	1/30	3/30	1/30	5.6 a	10.1 c
506	100	25.6 ab	1/30	1/30	2/30	4.4 a	7.6 b
507	250	18.0 a	0/30	1/30	1/30	2.2 a	7.6 b
508	500	17.0 a	0/30	0/30	0/30	0 a	7.1 b
509	2500	22.3 a	0/30	0/30	0/30	0 a	7.4 b

* Values followed by the same letter are not statistically different by Duncan Multiple Range Test at 0.05.

Table III. Extrusion and initiation on media varying in gellan gum concentration.

Media	Gelrite	Extrusion	Seed source and initiation			
	g L^{-1}	Average* (%)	WV–I_2	UC7–1037	UC10–27	Average* (%)
504	2.00	36.7 abcd	2/30	1/30	6/30	10.0 b
531	0	21.1 a	0/30	1/30	3/30	4.4 ab
532	0.25	25.6 ab	0/30	0/30	2/30	2.2 a
533	0.4	26.7 abc	0/30	1/30	4/30	5.6 ab
534	1.0	42.2 cd	1/30	0/30	7/30	8.9 b
505	2.0	50.0 d	5/30	3/30	10/30	20.0 c
535	4.0	41.1 bcd	1/30	1/30	0/30	5.6 ab

* Values followed by the same letter are not statistically different by Duncan Multiple Range Test at 0.05.

Table IV. Initiation rates on medium 505 and 539 and six month culture survival for ten open-pollinated families initiated during summer, 1995.

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

3.3. Initiation rates and culture survival for multiple families

Table IV shows a summary of initiation responses for two media, 505 and 539 (*table I*), varying in gellan gum content of 2.0 and 1.6 g L^{-1}, respectively. For ten open-pollinated families initiation averaged 16%. All families initiated cultures with rates ranging from 3–33%. After six months of transfers every two weeks, 78 of 98 cultures had died. Only 20% of the cultures survived, representing six of the ten starting families.

4. DISCUSSION

For SE Technology to become commercially successful it has to be integrated with breeding programs and has to be successful with a variety of genotypes. To work most effectively with the "leading edge of the breeding program", Timmis (1998) concluded that SE Technology needs to increase the efficiency of ESM (embryogenic tissue) establishment [23].

AC was shown to increase zygotic embryo extrusion from ovules. Somatic embryos formed and began to multiply, but in

the presence of AC > 100 mg L^{-1}, growth and multiplication often stopped. This inhibition was partially overcome by supplementing media with extra Cu and Zn to compensate for the adsorption of these elements by AC. Copper and zinc are essential elements and are required for plant growth. Both metals are present in growing tissue as structural chelates or metalloproteins and are bound to many essential enzymes [4]. Even with the supplementation of Cu and Zn, initiation was maximized only when AC was reduced to levels below 100 mg L^{-1}. One possible explanation is that stimulatory compounds are produced by the female gametophyte during the initiation process. As media AC levels increase, these compounds are removed and unavailable during initiation.

When this research began, initiation rates for LP were often below 1%. Our approach was to study natural embryo development and changes in medium over time. We expected that somatic embryo quantity and quality would improve through imitation of the hormonal, nutritional, and physical environment during zygotic embryo development. Medium 505 was developed through the use of ovule osmotic profile research [15], modeling AC uptake of 2,4-D [24] and research to understand the effect of pH and AC uptake on mineral availability [17, 25]. When tested with ten open-pollinated families, medium 505 initiated cultures at rates ranging from 3 to 33%.

Maintenance of embryogenic tissue from 98 initiations (ovules showing visible somatic embryos) over six months showed a loss of 4/5 cultures. While all of these initiations showed visible somatic embryos initially, many did not grow when transferred to the multiplication medium. In addition, another fraction of the cultures grew poorly for several subcultures and then died. A third fraction of cultures grew well for several subcultures, declined over time, and stopped growing. Literature on conifer SE occasionally mentions the loss of lines during maintenance or the inability to obtain stable lines. However, the magnitude of this problem is rarely quantified or discussed. Tautorus et al. (1991) indicated that 50% of the *Picea mariana* suspension cultures tested were discarded due to browning [21]. Loss of LP culture lines after initiation is a significant barrier that needs to be overcome for the commercial use of SE Technology.

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