Embryogenic Tissue Initiation in Loblolly Pine (*Pinus Taeda* L.)

Gerald S. Pullman

1 Introduction

Somatic embryogenesis (SE) technology has the potential to be the lowest-cost method to rapidly produce large numbers of high-value seedlings with desired characteristics for plantation forestry. SE is expected to play an important role in the future to increase forest productivity, sustainability and uniformity. SE technology has the advantages of: (1) shortening time to produce desired planting stock, (2) allowing control of genetic variation, (3) permitting commercial production of hybrids, and (4) facilitating genetic engineering efforts for desirable traits.

Since the first reports of somatic embryogenesis in *Picea abies* and *Larix decidua in* 1985 (Chalupa 1985; Hackman and von Arnold 1985; Nagmani and Bonga 1985), many different coniferous species have shown the ability to produce embryogenic tissue. At least 27 *Pinus* species are reported to go through SE (Pullman and Bucalo 2011). However, it should be emphasized that SE only works well with a few species. Often, even for the most responsive species, initiation frequency is low, many desired seed sources are recalcitrant, culture survival is low and/or embryo maturation often stops prematurely resulting in slow initial growth and low germination percentages. These difficulties raise the costs of somatic seedlings produced from successfully initiated genotypes.

Loblolly pine (*Pinus taeda* L.) is the most commercially important tree species in the Southeastern US and the second most common species in the US (Nix 2013). One to 1.5 billion trees are planted annually across the Southern USA

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(Schultz 1999). Since pine plantations in the South are expected to increase both in total area and silvicultural intensity, methods to provide the best planting stock will become increasingly important (Fox et al. 2007; Huggett et al. 2013).

Conifer SE proceeds through four steps: initiation, multiplication, maturation and germination and cryopreservation when storage of cultures is desired (Pullman et al. 2003a). This report will focus on the initiation step. The first report of SE in loblolly pine occurred in 1987 (Gupta and Durzan 1987). Since then many reports and patents on loblolly pine initiation have been published (Pullman and Webb 1994; Becwar and Pullman 1995; Pullman and Johnson 2002; Pullman et al. 2003a, c, d, 2005b, c, 2006, 2008, 2009, 2015; Pullman and Bucalo 2011; Pullman and Bucalo 2014).

As ET grows and somatic embryos develop in vitro, hormonal, nutritional and environmental conditions must be provided by the medium. Therefore, duplication of the seed hormonal, nutritional and environmental conditions found in vivo is likely to improve ET initiation or somatic embryo growth and development.

2 Natural and Somatic Embryogenesis

Natural zygotic embryogenesis starts with a fertilized egg and ends with a germinated plant (Gifford and Foster 1989). Conifer embryos arise from a single fertilization, creating a diploid embryo that develops in a haploid megagametophyte (Dogra 1967; Singh 1978; Nagmani et al. 1995). Conifer embryos grow and develop inside a megagametophyte 'corrosion cavity', a space that enlarges as the suspensor lengthens and pushes the embryo deeper into the seed. Programmed death of cells adjacent to the embryo provides nutrients for growth (Durzan 2012).

Multiple zygotic embryos usually occur in early-stage seeds of conifers and may form through two processes. In 'simple embryony' egg cells in different archegonia are fertilized by different pollen grains forming different genotypes. A process called 'cleavage polyembryony' usually follows in *Pinus*, where the immature embryos are multiplied. Loblolly pine seeds have 1–4 archegonia, each containing an egg cell (Fig. 1a). Fertilization can occur in one or more archegonia (simple polyembryony). Fertilized embryos in the seed divide into four embryos (cleavage polyembryony) so that up to 16 embryos may form within each seed (Fig. 1b). After simple or both types of embryony, one embryo becomes dominant and continues to grow (Fig. 1c). Subordinate embryos usually do not develop further but persist briefly in the ovule and appear to be the initiating material for SE in loblolly pine (Becwar et al. 1990, 1991; Becwar and Pullman 1995). MacKay et al. (2001) found that the number of zygotic embryos per seed may be a driver of initiation and could be a useful indicator of initiation potential.

During SE somatic cells from the plant reprogram to form somatic embryos. Hormonal and nonhormonal inducers can be used to promote the somatic embryogenic transition (Fehr 2003). Nonhormonal inducers are often stress factors

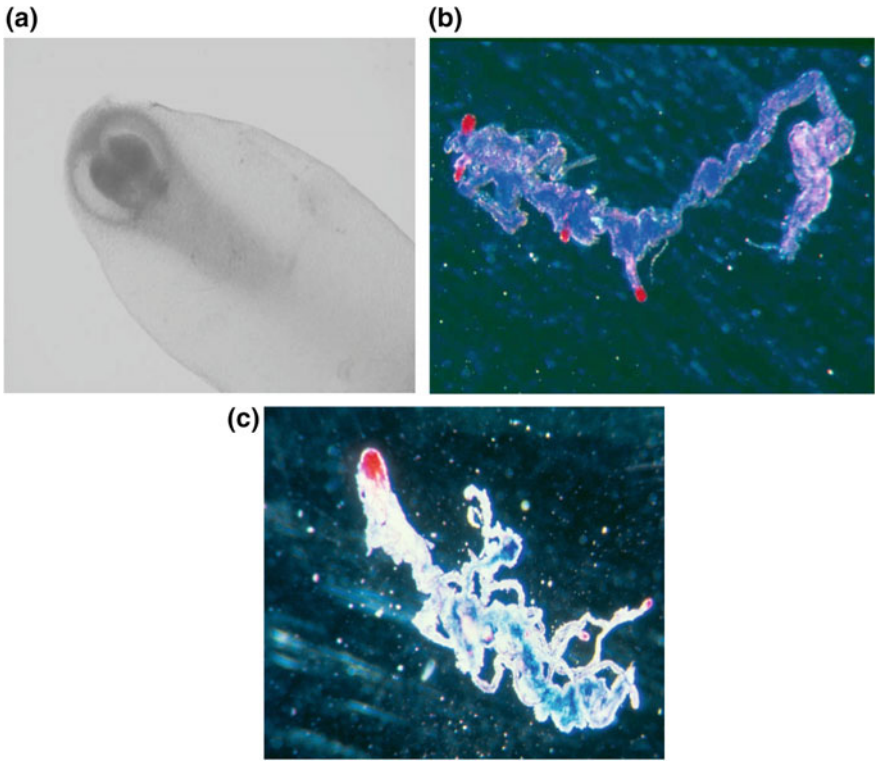

Fig. 1 Natural zygotic embryogenesis in *Pinus taeda*. **a** Megagametophyte with two archegonia visible shortly after fertilization. **b** Polyembryony several weeks after fertilization. Multiple early-stage zygotic embryos are visible resulting from simple and or cleavage polyembryony. Double-stained with acetocarmine and Evans blue (Gupta and Holmstrom 2005). **c** As development continues, one embryo becomes dominant and the subordinate embryos slowly die. Tissue stained with acetocarmine and Evans blue. Reproduced from Pullman and Bucalo (2014) with permission from Springer

and include osmotic shock, culture medium dehydration, water stress, heavy metal ions, altered culture medium pH, heat or cold shock, hypoxia, antibiotics, ultraviolet radiation, and some mechanical or chemical treatments (Zavattieri et al. 2010, Fehr 2003). Stress, in particular oxidative stress, appears to be an important initiator of SE (Fehr 2003). 2,4-dichlorophenoxyacetic acid (2,4-D) which is one of the most effective and commonly used initiators of SE appears to function as an oxidative stress activator. 2,4-D may act by increasing auxin activity and simultaneously increasing stress responses (Fehr 2003).

3 Materials

A. Seed (collected at specific developmental stages).
B. Media for *P. taeda*: initiation (2785, 2880), capture and maintenance (1250). Components are shown in Table 1.
C. Sterilizing solutions: 10% Liqui-Nox with 0.2% Tween 20; 20% H_2O_2.
D. Chemical reagents: reagent alcohol (70%).
E. Consumable supplies: scalpel blades (sterile), pipettes (10, 50 mL), vacuum filters (0.2 mm, 250 mL), syringe filter (0.2, 13 mm) Costar #3526 Well Culture Cluster Plates and Parafilm.

Table 1 Media components for loblolly pine initiation and capture

Media and components (mg/l)				
	1133	1250	2785	2880
NH_4NO_3	603.8	603.8	200.0	200.0
KNO_3	909.9	909.9	909.9	909.9
KH_2PO_4	136.1	136.1	136.1	136.1
$Ca(NO_3)_2$•$4H_2O$	236.2	236.2	236.2	236.2
$MgSO_4$•$7H_2O$	246.5	246.5	246.5	246.5
$Mg(NO_3)_2$•$6H_2O$	256.5	256.5	256.5	256.5
$MgCl_2$•$6H_2O$	101.7	101.7	101.7	101.7
KI	4.15	4.15	4.15	4.15
H_3BO_3	15.5	15.5	15.5	15.5
$MnSO_4$•H_2O	10.5	10.5	10.5	10.5
$ZnSO_4$•$7H_2O$	14.4	14.4	14.668	14.668
Na_2MoO_4•$2H_2O$	0.125	0.125	0.125	0.125
$CuSO_4$•$5H_2O$	0.125	0.125	0.1725	0.1725
$COCl_2$•$6H_2O$	0.125	0.125	0.125	0.125
$AgNO_3$	–	–	3.398	3.398
$FeSO_4$•$7H_2O$	6.95	6.95	13.9	13.9
Na_2EDTA	9.33	9.33	18.65	18.65
Maltose	–	–	15,000	15,000
Sucrose	30,000	30,000	–	–
Myo-inositol	1000	1000	20,000	20,000
Casamino acids	500	500	500	500
L-glutamine[a]	450	450	450	450
Thiamine•HCl	1.0	1.0	1.0	1.0
Pyridoxine•HCl	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5
Glycine	2.0	2.0	2.0	2.0
D-xylose	–	–	100	100

(continued)

Table 1 (continued)

Media and components (mg/l)				
	1133	1250	2785	2880
MES	–	250	250	250
Biotin	–	0.05	0.05	0.05
Folic acid	–	0.5	0.5	0.5
Vitamin B_{12}[a]	–	–	0.1	0.1
Vitamin E[a]	–	–	0.1	0.1
α-ketoglutaric acid[a]	–	–	100	100
Sodium thiosulfate			1.0 mM	1.0 mM
NAA	–	–	2.0	0.3
2,4-D	1.1	1.1	–	–
BAP	0.45	0.45	0.63	0.63
Kinetin	0.43	0.43	0.61	0.61
Activated charcoal	–	–	50	50
Abscisic acid[1]	1.3	1.3	–	9.0
24-epibrassinolide[a]	–	–	2.0 μM	2.0 μM
Gelrite	–	2500	2000	–
pH	5.7	5.7	5.7	5.7

[a]Filter-sterilized stock solution was added after autoclaving and cooling to 55–60 °C

4 Initiation of Embryogenic Tissue

4.1 Cone and Embryo Stage Collection

Somatic embryos can be grown from immature isolated zygotic embryo explants (Becwar et al. 1990), immature megagametophytes (Pullman and Bucalo 2011; Gupta 2016) or excised mature embryos (Tang et al. 2001). The most success has occurred with immature megagametophytes isolated from immature cones from breeding programs to initiate an ET culture or line. Open or control-pollinated cones are collected in early July when immature embryo stages inside the megagametophyte range from 2 to 4 (Pullman and Webb 1994; Cairney and Pullman 2007; Fig. 2). Cones are shipped on ice, received within 24–48 h and may be stored in plastic bags at 4–5 °C for several weeks until processed.

4.2 Initiation Medium Preparation

Medium is prepared, pH adjusted to 5.7 with KOH or HCl after addition of all ingredients except gelling agent and filter-sterilized materials then autoclaved at 121 °C for 20 min. Filter-sterilized solutions of L-glutamine, 24-epibrassinolide

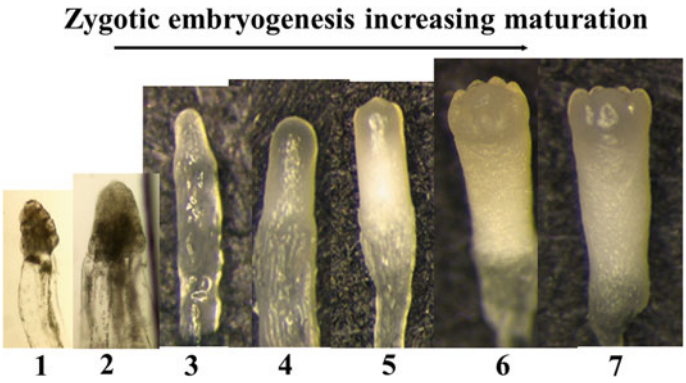

Fig. 2 Developmental stages for zygotic embryogenesis in loblolly pine. Based on Pullman and Webb (1994). Adapted from Cairney and Pullman (2007)

(E1641, Sigma-Aldrich, St. Louis, MO or E244, PhytoTechnology Laboratories, Showcase Mission, KS), and ABA are added to medium cooled to about 55 °C. Acid-washed tissue culture tested activated carbon (AC) (C9157) is purchased from Sigma-Aldrich. Epibrassinolide stock solutions are prepared in absolute ethanol (Aaper Alcohol and Chemical Co.). Solubility is about 3 mg/ml and care should be taken to minimize ethanol medium content to avoid reduced ET growth.

4.3 Seed Sterilization and Explant Preparation

Cones are cut open, and seeds removed, washed in running cold tap water for 10 min, agitated in 10% Liqui-Nox (detergent) with 2 mL Tween 20/L for 10 min, and rinsed in running tap water for 30 min. Seeds are agitated aseptically in 20% H_2O_2 for 10 min and rinsed five times for 5 min with sterile deionized water (Pullman et al. 2005c, 2015).

4.4 Aseptic Dissection, Explant Placement and Liquid Overlay Addition

The seed coat, integuments and nucelli are removed. The megagametophyte containing the embryo(s) is placed onto 2 mL of initiation medium 2785 contained in individual wells of Costar #3526 Well Culture Cluster Plates. Plates are wrapped in two layers of Parafilm and incubated at 23–25 °C in the dark. After 14 days, 0.25 mL of medium 2880 (Table 1) is added (Pullman and Skryabina 2007;

Pullman et al. 2015). The liquid overlay contains fresh medium, ABA, reduced NAA and functions to refresh medium contents, adjust pH, and expose extruding tissue to ABA.

4.5 Embryogenic Tissue Evaluation

Multiple points of ET initiation are often present on an explant. A typical sequence of initiation from immature zygotic embryos is shown in Fig. 3 and described in more detail by Becwar and Pullman (1995). *P. taeda* initiation occurs in three steps: extrusion at 1–4 weeks when most often subordinate zygotic embryos expand out of the megagametophyte micropylar end; cell proliferation and formation of a mass of ET (embryo suspensor mass). Initiation is evaluated after nine weeks and ET is transferred to medium 1250 (Table 1).

4.6 Embryogenic Tissue Capture and Maintenance

Tissue weights are tracked over three two-week subcultures on medium 1250, and an initiation is considered "captured" when it reaches 200 mg. A target weight of 200 mg was selected based on observations where captured cultures reaching this mass tended to continue growth while cultures of less weight had a greater chance of growth decline. About half of the new initiations reach 200 mg. The remaining 50% do not grow although ET formed but stopped growth within several months. During capture and maintenance transfers ET clumps are kept small (about 0.5 cm diameters) to maximize surface area where tissue grows most rapidly. Old, brown and dying ET in the center of larger clumps should be removed along with non-ET forming hard or green callus. This selection process is important to maintain ET as the culture ages.

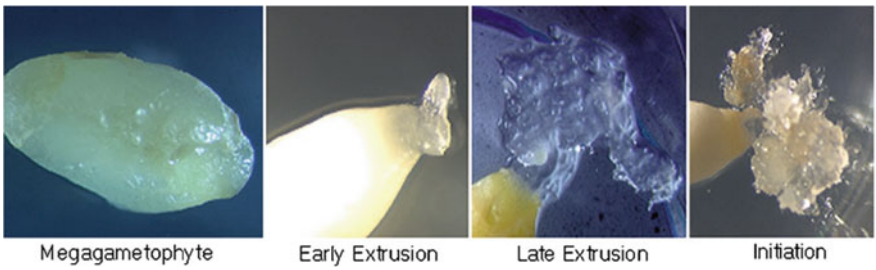

Fig. 3 Typical sequence of embryogenic tissue initiation in loblolly pine. Reproduced from Pullman et al. (2003d) with permission from Springer

5 Discussion

When research began, initiation rates for loblolly pine were often below 1%. Early improvements occurred through combinations of optimal embryo stages, half-strength P6 salts, ovule osmotic profile research, modeling AC uptake of 2,4-D and research to understand the effect of pH and AC on mineral availability (Teasdale et al. 1986, Pullman and Johnson 2002). Many improvements in loblolly pine initiation over the past 30 years have resulted from careful study of the developing seed and embryo (Pullman and Bucalo 2014, Xu et al. 1997, Cairney et al. 1999, 2000). Medium supplements and environmental conditions are available to improve ET initiation and somatic embryo development that have resulted from analytical studies of seed tissues, the seed environment and gene expression in the megagametophyte, zygotic embryos and somatic embryos.

Choice of explant. Immature and mature zygotic embryos have been used to initiate loblolly pine ET (Becwar et al. 1990; Becwar and Pullman 1995; Tang et al. 1998; Tang et al. 2001; Gupta 2016). However, initiation using whole megagametophytes containing optimum embryo stages has been the explant of choice due to ease of dissection. ET initiation has been found to correlate highly with the immature embryo stage within the megagametophyte and greatest initiation occurring from precotyledonary embryos at stages 2–4 (Becwar et al. 1990; Pullman and Johnson 2002; Pullman et al. 2003a, b, d). The staging system of Pullman and Webb (1994) is used to evaluate zygotic and somatic embryos. This system helps to understand variation in stage due to mother tree, location and time. Cone cold storage (4°) also can assist in obtaining target stages by allowing stage 1 embryos to slowly grow while in storage.

Initiation of embryogenic tissue. Extruded zygotic embryos have the same appearance as somatic embryos and cannot easily be distinguished except by observations of continued growth. Researchers have occasionally mistaken the zygotic extrusion process for ET and reported high initiation rates. Successful initiations will show ET forming a mass of proliferating cells and embryos that increase over time originating from the extruded zygotic embryos.

The ET frequently initiates from cell division and proliferation in the suspensor region near the interface of the suspensor cells and the embryo proper (sometimes called the embryo head) (Becwar et al. 1991; Becwar and Pullman 1995). The terms"embryonal suspensor masses" and "somatic polyembryogenesis" have been used to describe, respectively proliferating embryogenic cultures of loblolly pine and other conifers, and the in vitro embryo formation process in cultures (Gupta and Durzan 1987). Embryogenic tissue can be initiated from both dominant and subdominant zygotic embryos so that a culture may contain more than one genotype (Becwar et al. 1991).

Recently an interesting hypothesis was reported that ET from subordinate embryos undergoing cleavage embryony after the dominant embryo has formed may be inferior to ET developed prior to dominant embryo formation (Klimaszewska et al. 2007; Abrahamsson et al. 2017). ET lines from subordinate

embryos may carry forward degeneration patterns resulting from the beginning of programmed cell death that cause abnormalities in subsequent cotyledonary embryo development. Gupta (2016) recently reported a method for initiation from megagametophytes prior to dominant embryo formation that showed high initiation rates and may overcome this problem. In this method megagametophytes collected shortly after fertilization prior to dominant embryo formation were simply dissected about one-eighth from the micropylar end and cultured on initiation medium. Further studies are needed to understand and compare the effects on embryo development of initiation and cleavage polyembryony from subordinate, predominant and dominant embryos.

Maternal and paternal effects on initiation. Paternal and maternal effects on initiation in loblolly pine were examined by performing a diallel mating and following the extrusion and initiation frequencies of the resulting families, compared with open pollinated families (MacKay et al. 2006). Using reciprocal crosses (A × B and B × A), both mother-tree and pollen parent had significant effects on initiation frequency (Mackay et al. 2006). One tree, which was recalcitrant in culture as a mother tree, produced high initiation rates when used as a pollen parent. Certain mother trees gave high initiation with all of the pollen parents. The work showed initiation could be increased 46% by careful selection of mother and father parent trees.

Plant hormones and plant growth regulators. Six groups of plant hormones and plant growth regulators (PGRs) that function together to regulate plant growth and development were examined. All are known to be present in conifer seed tissues during early seed development.

Abscisic acid. Abscisic acid (ABA) is well known to regulate zygotic and somatic embryo maturation in both angiosperms and gymnosperms (Rai et al. 2011). ABA is produced by the megagametophyte and moves to the developing embryo. When the megagametophyte is absent in vitro, ABA must come from the medium. Kapik et al. (1995) measured ABA levels in loblolly pine zygotic tissues using an indirect ELISA method. When calculated on a micromole basis, peaks occurred in mid-development and during late embryo development. However, the presence of ABA throughout embryo development including early stages suggested ABA may improve ET initiation. Several research groups tested this hypothesis in *P. taeda* and other species and found increased initiation when ABA was added to the medium (Aitken-Christie and Parkes 1996; Handley 1997, 1999; Pullman and Skryabina 2007; Pullman et al. 2003c, 2009, 2016). The addition of 3.7 µM ABA, 20 µM $AgNO_3$ (see ethylene section) and optimization of cytokinin levels almost tripled initiation across 32 seed families (Pullman et al. 2003c). ABA also increased loblolly pine ET growth in maintenance medium and after retrieval of cryopreserved cultures (Becwar and Krueger 2004; Pullman et al. 2005b).

Auxins and cytokinins. Optimal concentrations of auxins and cytokinins in the form of man-made PGRs are usually determined through empirical tests or adopted from the literature. NAA at 2 mg/l was found to work well for loblolly pine (Pullman and Johnson 2002). Cytokinin concentrations were optimized at 0.63 mg/l BAP and 0.61 mg/l kinetin in the presence of 50 mg/l AC (Pullman et al. 2003c).

Brassinosteroids. Brassinosteroids (BRs) were discovered recently and are involved in numerous plant processes. BRs in seeds have diverse tissue-specific and species-specific effects on cell elongation, division and differentiation, reproductive biology, senescence, the stimulation of ethylene production, and an increase in resistance to abiotic stress (Brosa 1999; Clouse and Sasse 1998; Clouse 2001). With analytical information that BRs are found in gymnosperms including seeds, tests found increased ET initiation when media was supplemented with brassinolide or 24-epibrassinolide (Pullman et al. 2003d; Malabadi and Nataraja 2007; Pullman et al. 2009; Ma et al. 2012; Pullman et al. 2016). Brassinolide at 0.1 μM improved initiation rates in loblolly pine from 15.0 to 30.1%, increased weight of loblolly pine ET tissue by 66% and stimulated initiation in recalcitrant families (Pullman et al. 2003d). Recently brassinolide has been difficult to obtain and 2.0 μM 24-epibrassinolide has been substituted (Pullman et al. 2015).

Ethylene. Ethylene can be produced by almost all parts of plants and is known to have significant effects on plant growth in vitro. Ethylene may act as either a growth promoter or inhibitor depending on the species (Biddington 1992). Ethylene was shown to reduce somatic ET growth in *Picea glauca* suspension cultures (Kumar et al. 1989). Preliminary analytical tests showed presence of ethylene in our culture containers (Pullman et al. 2003c). We therefore tested for effects of ethylene and ethylene inhibitors on loblolly pine initiation. Several reports have shown improved embryogenesis when silver nitrate, a strong ethylene action inhibitor, was added to the medium (Beyer 1976; Auboiron et al. 1990; Roustan et al. 1989, 1990; Li and Huang 1996). When 20 μM silver nitrate was added to the medium, loblolly pine ET initiation increased (Pullman et al. 2003c). It should also be noted that AC, also present in the medium, functions as an ethylene adsorbent (Thomas 2008).

Gibberellins. Gibberellins (GAs) are present in fruits and seeds and have been reported to both increase and decrease SE in angiosperms (Rademacher 2000; Rudus et al. 2000). Because GAs are known to be present in conifer seeds (Kong et al. 1997) we hypothesized that GAs may improve ET initiation for loblolly pine. In our first experiment the opposite occurred, GA_3 decreased ET initiation. With reduced initiation from added GA_3 and increased initiation with added ABA (Pullman et al. 2003c), we hypothesized reductions in endogenous GAs content to decrease the GA: ABA ratio would improve initiation. Paclobutrazol, an inhibitor of a reaction in the gibberellin synthesis pathway, improved ET initiation for loblolly pine, slash pine (*Pinus elliottii*), Douglas fir (*Pseudotsuga menziesii*) and Norway spruce (*P. abies*) (Pullman et al. 2005c). Using 0.33–1.0 mg/l paclobutrazol, initiation percentages in loblolly pine were improved from 37.7 to 44.2%. Other gibberellin inhibitors, effective at different points in the gibberellin pathway also showed statistically significant increases in ET initiation (Pullman et al. 2005c). Studies on meristem cells show GAs are excluded or kept low in meristem initials and may need to be low for formation of somatic embryos (Sakamoto et al. 2001). Paclobutrazol (95.8% active ingredients, Duchefa, Netherlands) stock solution slurries of 1 mg/ml are vortexed and rapidly added to the medium prior to autoclaving. Paclobutrazol has a solubility of 35 mg L^{-1}. Paclobutrazol is not

added to the current recommended initiation medium but may be useful for recalcitrant seed sources.

Nutritional components. The conifer embryo grows and develops within the megagametophyte in a corrosion cavity where secreted fluids nourish the embryo (Carman et al. 2005). Nutritional components of the megagametophyte or more finely, the embryo-megagametophyte interface, are of interest to help develop stage-specific SE growth media. The nutritional components and their stage-specific physiological concentrations are slowly becoming known.

Minerals. Teasdale et al. (1986) used mineral analysis of loblolly pine seed to formulate P6 medium for non-embryogenic suspension cultures of loblolly pine. The medium generally contained high concentrations of micronutrients and magnesium and low calcium. Hi iodide, borate and zinc were found to be beneficial to growth. Half-strength P6 salts has worked well for loblolly pine ET initiation and is used in our research. Pullman and Buchanan (2003) analyzed stage-specific *P. taeda* embryo and megagametophyte tissues for 14 key metals. The analytical data assisted in medium development for embryo maturation (Pullman et al. 2003b). Loblolly pine initiation has also been reported using other salt recipes: DCR (Becwar et al. 1990), WV5 (Coke 1996), LOB (Tang et al. 1998) and TX (Denchev et al. 2011).

O*rganic acids*. Organic acids are important in plant metabolism and can occur in large amounts as free anions altering tissue water potential (Taiz and Zeiger 2010). Several organic acids are present in all plants in the citric acid cycle. When 26 organic acids were analyzed in loblolly pine seed tissues, five showed statistically significant increases in early-stage somatic embryo growth when added to medium at approximate physiological concentrations (Pullman and Buchanan 2006; Pullman et al. 2006). α-ketoglutaric acid, pyruvic acid and succinic acid improved ET initiation when alone or combined. The combination of these three amino acids and vitamins B_{12} and E showed 36.3% ET initiation across four loblolly pine seed sources compared to 27.3% initiation in a control medium.

Sugars. Carbohydrates play important roles providing energy and carbon for biosynthesis, as osmotic agents, in seed desiccation and cold tolerance, and as developmental regulators controlling gene expression (Iraqui and Tremblay 2001). Carbohydrates can accumulate in large amounts in seeds as deposited or dissolved free molecules. Pullman and Buchanan (2008) analyzed loblolly pine stage-specific embryo and megagametophyte tissues for starch and 18 sugars. When 17 sugars were screened at approximate physiological concentrations for effect on early-stage somatic embryo growth, D-xylose or D-*chiro*-inositol increased growth (Pullman et al. 2008). Medium supplementation with D-xylose or D-*chiro*-inositol increased loblolly pine initiation averages by +6.5% or +7.3%, respectively. Profiles of maltose showed high concentrations during early embryo development with a disappearance as a major shift in embryo development occurred after stage 9.1. This observation supported use of maltose as the main carbon source for initiation (Pullman and Johnson 2002). While not present in loblolly pine seeds, lactose increased ET culture initiation when used as a carbon source (Denchev et al. 2011).

Vitamins. Vitamins function as cofactors for essential metabolic reactions. Thiamine hydrochloride (Vitamin B_1), pyridoxine (Vitamin B_6), and nicotinic acid (niacin) are often present in plant tissue culture media and are common in conifer SE media. Benefits can occur from use of other vitamins including ascorbic acid (Vitamin C), biotin (Vitamin H), choline chloride (Vitamin B_4), cyanocobalamin (Vitamin B_{12}), folic acid (Vitamin M), pantothenic acid (Vitamin B_5), para-aminobenzoic acid, riboflavin (Vitamin B_2) or tocopherol (Vitamin E) (Bourgin and Nitsch 1967; Kao and Michayluk 1975; Dodds and Roberts 1995). When organic acids were profiled in seed tissues (Pullman and Buchanan 2006), ascorbic acid and nicotinic acid were also found in early embryo stages. With this observation, Pullman et al. (2005b, 2006) tested mixtures of biotin and folic acid or nine vitamins for effect on growth of early-stage somatic embryos. Biotin, folic acid, Vitamin B_{12} and Vitamin E alone or combined increased growth and prompted tests on ET initiation. These vitamins alone or combined increased ET initiation (Pullman et al. 2005b, 2006). Initiation increased from 22.5 to 38.5% using 12 loblolly pine families and medium supplemented with 2(n-morpholino) ethanesulphonic acid (MES, see pH section below), biotin and folic acid (Pullman et al. 2005b).

Duplication of physical seed conditions in vivo. Internal seed conditions other than nutrition and hormonal factors can influence embryo growth and development. Gas concentrations of O_2, CO_2 and ethylene, movement of water, H^+ concentration (pH), redox potential and dynamics of nutrients, hormones and waste products are a few of the factors likely to affect seed and embryo development and ET initiation.

Water potential. Water potential (Ψ) conditions appear to control embryo development for many plant species (Bradford 1994). Ψ can be used to describe the tendency of water to move from areas of higher Ψ to areas of lower Ψ. While moving, water may carry dissolved nutritional components and thus regulate solute availability to the megagametophyte and developing embryos. Water relation parameters have been partially investigated for zygotic and somatic embryos of *P. taeda* (Dumont-BeBoux et al. 1996; Pullman 1997; Pullman and Johnson 2009b). These investigations showed that seed tissue Ψ values were much greater (measured in mmol/kg) than that measured in typical initiation media. This suggested that medium supplementation with osmoticants may improve initiation. Indeed, supplementation of initiation medium with 22.2 mM myo-inositol increased extrusion and proliferation (Li and Huang 1996) and 111 mM myo-inositol, raising medium osmolality about 120–130 mmol/kg, resulted in statistically significant increases in ET initiation (Pullman and Johnson 2002).

Activated carbon. AC is used in many tissue culture media. Benefits of AC are not well understood but may occur from adsorption of medium residual hormones, plant waste products, and toxic metabolites such as phenolic compounds, 5 hydroxy methyl-furfural and ethylene (Pan and van Staden 1998; Thomas 2008). Benefits may also occur from changes in medium nutrient and hormone dynamics as AC adsorbs component(s) or from change in endogenous hormones. Von Aderkas et al. (2002) quantified eight PGRs in ET of larch grown in media with or without 1% AC. AC caused a statistically significant increase in endogenous auxin.

Since AC may adsorb 95–99% of the hormones and PGRs present in medium, Pullman and Johnson (2002) tested initiation for loblolly pine on media with greatly increased PGRs combined with 2.5 g L^{-1} AC. Increased extrusion occurred when AC was added; however, only a few initiations resulted. Toering and Pullman (2005) tracked availability of radio-labeled 2,4-D in media. After adsorption, media with 2.5 g L^{-1} AC and 220 mg L^{-1} 2,4-D still contained too much 2,4-D with 12–17 mg L^{-1} available during much of the initiation period. Two approaches were suggested to improve initiation: (1) lower 2,4-D from 220 to 110 mg L^{-1} with 2.5 g L^{-1} AC; or (2) greatly reduce AC and combine with standard or slightly raised PGR levels similar to levels in media without AC. The second approach worked well when 50 mg/l AC was used and $CuSO_4$ $5H_2O$ was raised (Pullman and Johnson 2002). The high AC likely created a deficiency in Cu^{+2} by adsorbing most of the copper (Pullman and Johnson 2002; Van Winkle et al. 2003). Additional medium component adsorption studies have helped to develop effective media (Ebert and Taylor 1990; Nissen and Sutter 1990; Ebert et al. 1993; Pan and van Staden 1998; Van Winkle et al. 2003; Van Winkle and Pullman 2003, 2005; Toering and Pullman 2005; Pullman et al. 2005a).

pH. H^+ concentration (pH) controls many chemical reactions. Pullman and Johnson (2009a) measured pH of loblolly pine seed tissues. Megagametophytes measured pH 5.5 shortly after fertilization, about 6.1 at mid-development and 6.3–6.5 during late development. In contrast, embryo pH remained nearly constant at 7.0. Based on a logarithmic scale, a pH difference of 1.0 equals a tenfold difference in H^+ concentration. Measurements of pH 5.5 around the early-stage embryo suggested initiation media should target the same pH. Medium pH is known to change during tissue growth, dropping from ammonium usage and increasing from nitrate usage (Minocha 1987; Lulsdorf et al. 1992; Pullman et al. 2005b). Change in pH may also alter availability of ions and PGRs. Measurements of low medium pH at 4–4.5 during initiation suggested that maintaining the target pH may improve initiation. MES pH buffer agent and liquid medium added after 14 days provided pH control and increased initiation (Pullman et al. 2005b; Pullman and Skryabina 2007).

Liquid medium. Nutritional and hormonal components are delivered to the developing embryo by a surrounding aqueous film. Liquid medium advantages often include faster growth rates, lower variation, better visualization of tissues, and automation of cell suspension transfer. Adsorption of medium components differs in gelled vs. liquid media suggesting diffusion rates differ when medium is gelled (Ebert and Taylor 1990; Pullman et al. 2005a). Loblolly pine initiation media required reduction in NAA from 2 mg/l in gelled medium to 0.3 mg/l in liquid medium (Pullman and Skryabina 2007). Liquid overlays can be easily added to growing tissue to adjust pH, refresh components and/or add a new ingredient. When pH declined below target levels, liquid overlays added after 14 days containing 0.3 mg/l NAA brought pH back to desired levels and improved initiation +8.5% for high-value control-pollinated seed sources and +6.5 to +9.9% for open-pollinated and often recalcitrant seed sources (Pullman and Skryabina 2007).

Redox potential. Glutathione (GSH, reduced form)/glutathione disulfide (GSSG, oxidized form) and ascorbic acid (reduced form)/dehydroascorbate (oxidized form) are major redox pairs that control redox-state in a developing seed. Early-stage embryo development appears to occur best in a reducing environment while late-stage development occurs best in a more oxidizing environment (Stasolla 2010). Redox potential has been shown to modify embryo development in several plants including white spruce and the ratio of GSH: GSSG seems to be more important than the actual amounts of GSH and GSSG (Yeung et al. 2005).

Glutathione appears to be essential for SE, as silencing GSH biosynthetic pathways in wheat inhibited SE (Bossio et al. 2013). Expression of HBK3, a major embryogenesis control gene required for differentiation of proembryogenic masses in *P. abies* somatic embryos, was associated with ascorbate and glutathione metabolism (Belmonte and Stasolla 2009).

Pullman et al. (2015) found ASC and GSH in loblolly pine megagametophyte or zygotic embryos at low concentrations during stage 1, but DHA and GSSG were not present at all or were barely detectable. In vitro early-stage somatic embryo growth during ET initiation or maintenance may therefore benefit from addition of ASC, GSH or other non-toxic reducing agents.

Because high costs of GSH may prohibit its use, Pullman et al. (2015) investigated effects of low-cost anti-oxidants on ET growth or initiation. Sodium dithionite and sodium thiosulfate were effective reducing agents and increased early-stage somatic embryo growth and ET initiation for *P. taeda* and ET initiation for *P. menziesii*. Reducing agents increased loblolly pine initiation averages by 8–99% and *P. menziesii* initiation by 5–30% in trials over four years. Ascorbic acid, a combination of vitamins including the anti-oxidant tocopherol (vitamin E), or GSH increased *P. glauca*, *P. taeda* or *Araucaria angustifolia* ET proliferation or initiation (Stasolla and Yeung 1999; Pullman et al. 2006; Vieira et al. 2012).

5.1 Concluding Remarks

The loblolly pine initiation medium and practices presented were developed over 30 years. Many of the improvements were based on analytical studies of *P. taeda* developing seed, embryos and seed tissues. Most of the improvement concepts that have increased initiation in loblolly pine have also been shown to increase initiation for other species and therefore show promise for general use with coniferous species.

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