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Shri Mohan Jain · Pramod Gupta *Editors*

Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants

Volume I

Second Edition

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Preface

There is an increasing demand for various tree products as the world population continues to grow rapidly, leading to slow down in woody plant product supplies worldwide. To meet demand of every growing human population, there is a need to maintain continuous supply of woody products by increasing productivity of trees. This can be achieved by improving breeding of trees with better traits; however, conventional breeding methods are slow due to long life cycle of trees.

A basic strategy in tree improvement is to capture genetic gain through clonal propagation. Clonal propagation via organogenesis is being used for the production of selected elite individual trees. However, the methods are labour intensive, costly, and produce low volumes. Genetic gain can now be captured through somatic embryogenesis. Formation of embryos from somatic cells by a process resembling zygotic embryogenesis is one of the most important features of plants. It offers a potentially large-scale propagation system for superior clones. It has several additional advantages such as the ability to produce large numbers of plants, the potential for automation, the opportunities for synthetic seed, long-term storage, packaging, direct delivery systems and genetic manipulation.

Earlier, we edited a series on "Somatic Embryogenesis of Woody Plants", volumes 1–6, which provided reviews on somatic embryogenesis of important angiosperm and gymnosperm tree species. This series has become an excellent source of information for the researchers and students and did not provide "detailed protocols" for inducing somatic embryogenesis. Therefore, researchers may face difficulties in the initiation of somatic embryogenic cultures. The choice of explant is crucial for the initiation of embryogenic cultures.

This book is the second edition of previously published book entitled "Protocol for Somatic Embryogenesis in Woody Plants", 2005, and divided in two volumes. Both volumes include chapters on stepwise protocols of somatic embryogenesis of selected woody plants. This would enable both researchers and students to start somatic embryogenic cultures without too much alteration.

In Volume 1, each chapter provides information on initiation and maintenance of embryogenic cultures; somatic embryo development, maturation and germination; acclimatization and field transfer of somatic seedlings. Some chapters include

applications of somatic embryogenic cultures, e.g. **SE Fluidics System**, anther culture, manufactured seeds, cryopreservation and liquid cultures.

The invited authors are well known in somatic embryogenesis research, and they belong to industry, universities and research institutes. Each chapter has been extensively reviewed by other expertise before publication. We are grateful to all authors for their contribution to this book; and all reviewers reviewed chapters that have maintained high quality of the book.

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Pinus radiata (D. Don) Somatic Embryogenesis

Itziar A. Montalbán and Paloma Moncaleán

1 Introduction

Radiata pine (*Pinus radiata* D. Don) is one of the most widely planted exotic pine species in rainfall environments of the Southern hemisphere (Yan et al. 2006). Its fast growth has stimulated an exhaustive study of wood production, and the development of breeding programs (Espinel et al. 1995; Codesido and Fernández-López 2009). Although utility of in vitro organogenesis has been proven for clonal propagation of this species (Aitken-Christie et al. 1985), a limitation of this method is the high cost of the process for mass production commercially. Other systems to achieve in vitro propagation of *Pinus radiata* adult trees have been developed (Montalbán et al. 2013), but changes in the attributes of resulting plants have sometimes been observed and rejuvenation of the material has been transitory under in vitro conditions. Somatic embryogenesis (SE) has been the most important development for plant tissue culture, not only for mass propagation but also for enabling the implementation of biotechnological tools that can be used to increase the productivity and wood quality of plantation forestry. Therefore, many efforts have been made in the last years to develop and optimize SE systems that can be used in the breeding programs.

Somatic embryogenesis in *P. radiata* was first described by Smith et al. (1994) followed by improved protocols of different aspects of SE such as initiation (Hargreaves et al. 2009; Montalbán et al. 2012), maturation (Montalbán et al. 2010), cryopreservation (Hargreaves et al. 2002) and expression of genes (Aquea and Arce-Johnson 2008; García-Mendiguren et al. 2015). Modifications of the tissue culture media are likely to influence the success of SE initiation (Montalbán et al. 2012). However, few studies have focused on the impact of temperature

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(Kvaalen and Johnsen 2007). It is known that modifications in water availability, either by solute-imposed water stress or by physical restriction, will impact the development of embryonal masses (EMs) (Klimaszewska et al. 2000). Although the effect of different concentrations of gellan gum at maturation has been studied (Teyssier et al. 2011; Morel et al. 2014), the combination of different temperatures and water availability has not been previously tested at the initial stages of SE in conifers. As reviewed by Von Aderkas and Bonga (2000) and Neilson et al. (2010), it is clear that stress has the potential to induce or improve embryogenesis in species that have been considered recalcitrant.

Long periods of proliferation of the EMs can produce losses by contamination, somaclonal variation, or a decrease in their ability to generate embryos together with the high maintenance costs (Breton et al. 2006). One way to overcome this bottleneck is the cryopreservation of EMs; EMs are kept in liquid nitrogen because these low temperatures induce the synthesis of proteins that favours the conservation and subsequent viability of the EMs (Kong and von Aderkas 2011). However, this method presents some drawbacks such as: it is a complex technique comprising several stages (Gale et al. 2007); – it is an expensive process from the economic and technical point of view (Bomal and Tremblay 2000); it is necessary the presence of cryoprotectants that prevent the formation of ice crystals (Salaj et al. 2012); the most popular cryoprotectant is DMSO but is toxic (Arakawa et al. 1990) and may be the cause of genetic and epigenetic changes in tissues (Krajnakova et al. 2011). Nowadays, efficient and reproducible protocols for EMs cryopreservation have been described in Pinaceae (Lelu-Walter et al. 2008). However, cryopreservation of somatic embryos (Se) has been achieved for periods less than one month (Barra-Jiménez et al. 2015) in *Quercus* species, which does not guarantee long-term storage. Preliminary studies on *P. radiata* and other conifers (Hargreaves et al. 2004; Kong and Von Aderkas 2011), suggest that it is possible to develop simple alternative cryopreservation of Se at low temperatures maintaining their viability in the future.

An improved protocol for initiation of EMs, proliferation, somatic embryo maturation and germination as well as low temperature Se storage are presented in this chapter. Furthermore, recent studies focused on SE optimization in *Pinus radiata* are shown.

2 Initiation of Embryogenic Tissue

Cone collection and embryo stage assessment One-year-old green female cones, enclosing immature zygotic embryos of *Pinus radiata* at the precotyledonary stage (Montalbán et al. 2012), are collected and stored at 4 °C until processing. Cones are usually processed within one week, although they can be stored for more than one month with no detriment in SE initiation rates (Montalbán et al. 2015).

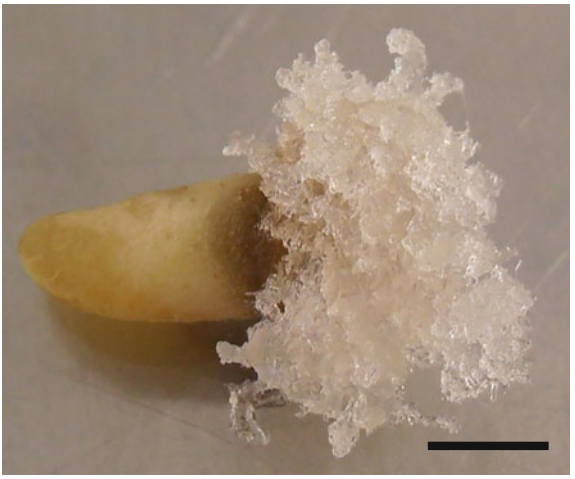

Fig. 1 Initiation of embryonal masses from *Pinus radiata* megagametophytes cultured at 23 °C on EDM (Walter et al. 2005), bar 0.2 cm

Seed sterilization Intact cones are sprayed with 70% (v/v) ethanol, split into quarters and all immature seeds dissected. Then, immature seeds are surface sterilized in H_2O_2 10% (v/v) plus two drops of Tween 20® for 8 min and then rinsed three times under sterile distilled H_2O in sterile conditions in the laminar flow unit. Seed coats are removed and whole megagametophytes containing immature embryos are excised out aseptically and placed horizontally onto the medium (Fig. 1).

Basal medium preparation Initiation of embryogenic tissue is usually carried out on EDM basal medium (Walter et al. 2005, Table 1) at 23 °C. The initiation medium contains 30 g L^{-1} sucrose, 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 2.7 μM benzyladenine (BA) and 3 g L^{-1} gelrite®. The pH is adjusted to 5.7, and the medium is sterilized at 121 °C for 20 min. After autoclaving,

Table 1 Constituents of *Pinus radiata* in vitro culture medium including salt components and organic additives. Note that hormonal supplements, carbohydrate source, activated charcoal or agar concentrations are specified in the text according to culture stage

Component	EDM	LP m
Inorganic salts	*Concentration* mg L^{-1}	
KNO_3	1431	1800
$MgSO_4 \cdot 7H_2O$	400	440
KH_2PO_4		675
$CaCL_2 \cdot 2H_2O$	25	
$Ca(NO_3)_2 \cdot 4H_2O$		835
NH_4NO_3		400
$NaNO_3$	310	
$NH_4H_2PO_4$	225	
KCl		
$MnSO_4 \cdot 4H_2O$	3.6	1

(continued)

Table 1 (continued)

Component	EDM	LP m
H_3BO_3	8	6.2
$ZnSO_4 \cdot 7H_2O$	25	8.6
KI	1	0.08
$CuSO_4 \cdot 5H_2O$	2.4	0.025
$Na_2MoO_4 \cdot H_2O$	0.2	0.25
$CoCl_2 \cdot 6H_2O$	0.2	0.025
$FeSO_4 \cdot 7H_2O$	30	30
$Na_2EDTA \cdot 2H_2O$	40	40
Vitamins		
Thiamine · HCl	5	0.1
Nicotinic acid	5	0.5
Pyridoxine · HCl	0.5	0.5
Myo-inositol	1000	100

filter-sterilized solutions (pH 5.7) of the following amino acids are added to partially cooled medium prior to dispensing into Petri dishes (90 × 9 × 20 mm): 550 mg L^{-1} L-glutamine, 525 mg L^{-1} asparagine, 175 mg L^{-1} arginine, 19.75 mg L^{-1} L-citrulline, 19 mg L^{-1} L-ornithine, 13.75 mg L^{-1} L-lysine, 10 mg L^{-1} L-alanine and 8.75 mg L^{-1} L-proline.

Culture conditions and incubations period
Cultures were maintained in the dark at 22 ± 1 °C for 4–8 weeks.

3 Embryonal Masses Evaluation

After 4–8 weeks on initiation medium, the number of initiated embryonal masses (3–5 mm in diameter) per Petri dish are evaluated.

4 Embryogenic Tissue Proliferation

Proliferating tissues are separated from the megagametophytes and subcultured to proliferation medium every 2 weeks. Initiation and proliferation medium only differ in the concentration of Gelrite®, being 3 g L^{-1} for the first and 4.5 g L^{-1} for the second. Following four periods of subculturing, actively growing embryogenic tissues are recorded as established cell lines (ECLs). Proliferation is carried out in darkness.

5 Somatic Embryo Maturation

The EMs are suspended in EDM (Table 1) broth (lacking growth regulators) and shaken vigorously by hand for several seconds. A 5 mL aliquot containing 80–90 mg of embryonal fresh mass is transferred to filter paper (Whatman no.2) in a Büchner funnel. A vacuum is applied for 10 s, and the filter paper with the attached tissue is transferred to maturation medium (Montalbán et al. 2010). The maturation medium contained the salt formulation of EDM (Table 1), 9 g L^{-1} gellan gum, 60 μM abscisic acid, 60 g L^{-1} sucrose and the amino acid mixture used for the initiation and maintenance of the EMs. Maturation is carried out in darkness.

6 Somatic Embryo Germination

After 15 weeks, Se (Fig. 2) are transferred to germination medium. This medium contains half-strength macronutrients LPm (Quoirin and Lepoivre 1977, as modified by Aitken-Christie et al. 1988) (Table 1) with 2 g L^{-1} of activated charcoal and 9.5 Difco agar. Petri dishes are tilted at a 45º angle with embryonic root caps pointing downwards and incubated under dim light for 7 days. Cultures are then maintained at a 16-h photoperiod at 100 μmol m^{-2} s^{-1} using cool white fluorescent tubes (TFL 58 W/33; Philips, France). Plantlets (Fig. 3) are subcultured onto fresh germination medium every 6 weeks. The whole in vitro SE process is carried out at 23 °C.

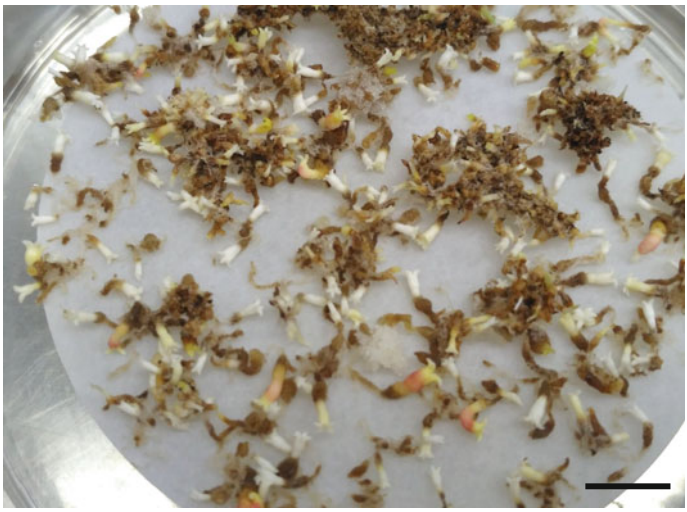

Fig. 2 Maturation of *Pinus radiata* somatic embryos cultured at 23 °C on EDM (Walter et al. 2005), bar 1.1 cm

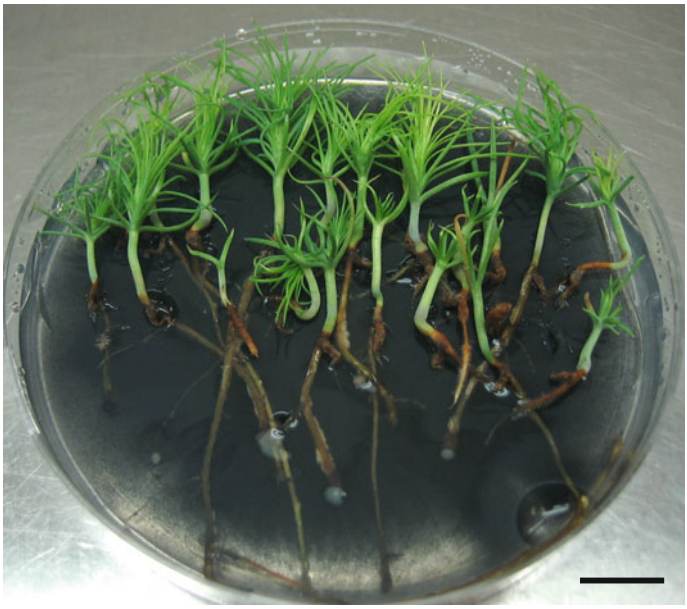

Fig. 3 Germination of *Pinus radiata* somatic embryos cultured at 23 °C on half-strength macronutrients LP (Quoirin and Lepoivre 1977, as modified by Aitken-Christie et al. 1988), bar 1.4 cm

7 Somatic Plantlet Acclimatization

After 14–16 weeks on the germination medium, the plantlets are transferred to sterile peat:perlite (2:1) and acclimatized in a greenhouse where the humidity is progressively decreased from 99 to 70% during the first month.

8 Abiotic Stress: A Way to Improve the Somatic Embryogenesis Process

In order to evaluate the effect of different physical and chemical conditions on radiata pine SE and to identify what initial stage of SE has the greatest impact on the success of embryogenesis, initiation was carried out in following the same methodology described in Sect. 2. Different concentrations of gellan gum were added prior to autoclaving to increase or reduce water availability in the medium (2, 3 or 4 g L^{-1} Gelrite®), and the explants were stored at 18, 23 or 28 °C (Fig. 4). In summary, nine different treatments were applied.

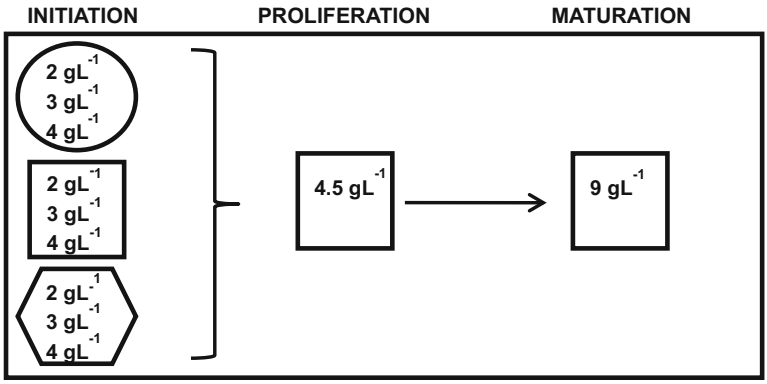

Fig. 4 Scheme of the experimental design, cultures were stored at initiation at three different temperatures: 18 °C (circle), 23 °C (square) or 28 °C (hexagon) and at three different agar concentrations (inside circles, squares and hexagons). The rest of the process was carried out under standard conditions

Statistically significant differences in the percentage of initiation among temperatures and gellan gum concentrations were found (García-Mendiguren et al. 2016).

When considering temperature alone, initiation percentages in explants induced at 28 °C were significantly lower (4%) than those induced at 18 or 23 °C (17–13%, respectively). With respect to gellan gum, megagametophytes cultured on medium containing 4 g L^{-1} gellan gum showed significantly higher initiation (16%) in comparison to those cultured at 2 and 3 g L^{-1}), which showed initiation values of 9% and 10%, respectively.

At the proliferation stage, statistically significant differences were identified only between temperatures (28 °C resulted in a significantly higher proliferation percentage (65%) when compared to explants initiated at 18 and 23 °C (35%). Regarding the number of Se per gram of EM, statistically significant differences were observed among initiation temperatures. ECLs initiated at 28 °C produced a significantly higher number of Se (486 Se g^{-1} EM) than those initiated at 23 °C (319 Se g^{-1} EM) (García-Mendiguren et al. 2016). Our results suggest that the initial conditions of the process positively impact the number of embryos produced several months later. Temperature presumably exerts a selective pressure in the early stages of embryogenesis and results in lower initiation rates but higher rates of proliferation and maturation (Fehér 2015). Although the different gellan gum concentrations tested show significant differences in water availability, this did not induce significant differences in the number of Se produced.

In summary, we observe a marked effect of initiation conditions on Se production, showing differences when that conditions are applied several months before. In light of the conclusions obtained in this study, initiation at 18 °C and 4 g L^{-1} gellan gum can be used to enhance the number of ECLs and thus enhance diversity within clonal plantations. On the other hand, incubation at 28 °C and the

addition of 2 g L^{-1} gellan gum at initiation increase the efficiency of the process and result in a larger number of clones from a selected cross in a genetic improvement program.

9 New Methods for Storing *Pinus radiata* Genetic Resources

P. radiata Se are placed onto a sterile Whatman filter (nº 2) and the filter laid on Petri dishes containing EDM (Table 1) (Walter et al. 2005) supplemented with 60 g L^{-1} sucrose and 9 g L^{-1} Gelrite®; after autoclaving the amino acid mixture of

Fig. 5 *Pinus radiata* somatic embryos after 12 months of storage at 4 °C, bar 0.4 cm

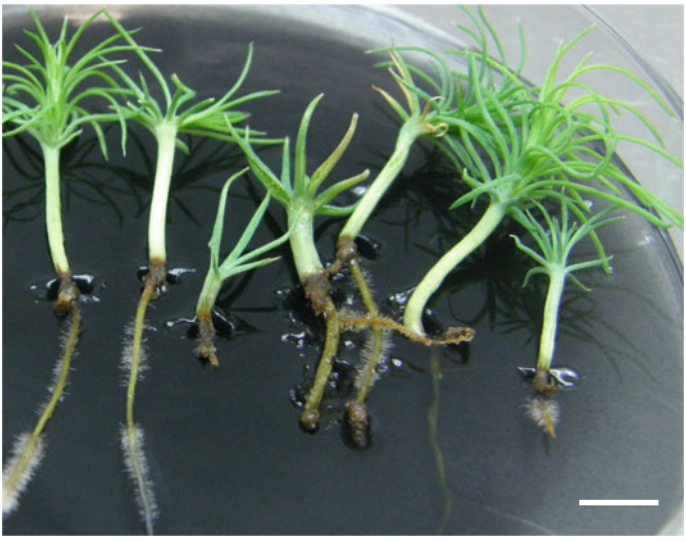

Fig. 6 Germination of *Pinus radiata* somatic embryos after 12 months of storage at 4 °C, bar 0.8 cm

the EDM medium (Table 1) is added. The Petri dishes are sealed with parafilm and can be stored at 4 °C for 1 year (Fig. 5). The percentage of germination is not affected by storage, improving the rates obtained in Se not conserved in cold (85%) (Fig. 6).

10 Research Prospects

Forestry productivity can be increased via the planting of high-value trees. Clonal propagation by somatic embryogenesis has the ability to enhance this amplification process and capture the benefits of breeding programs (Pullman et al. 2005) and it should be implemented with other technologies as cryopreservation of the embryonal masses (Park 2002) and/or somatic embryos. Our future researches activities are focused on corroborate the following hypotheses:

- Extreme environmental conditions during the early stages of somatic embryogenesis in *Pinus* spp. determine the adaptative characteristics of the somatic plants produced.
- The adaptive characteristics of the somatic plants of *P. radiata* are translated into differences in biochemical, molecular and physiological quantifiable characteristics, which could be used as early indicators of stress tolerance.
- The EMs and Se of *P. radiata* can be stored at 4, −20 and −80 °C minimizing the costs and use of cryoprotectans.

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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

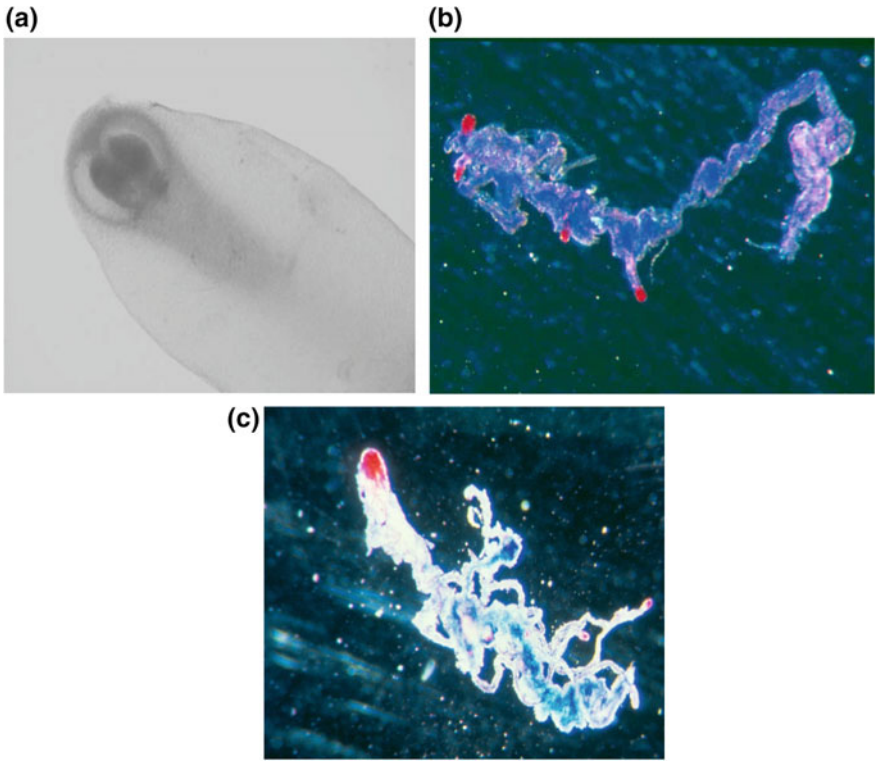

Fig. 1 Natural zygotic embryogenesis in *Pinus taeda*. **a** Megagametophyte with two archegonia visible shortly after fertilization. **b** Polyembryony several weeks after fertilization. Multiple early-stage zygotic embryos are visible resulting from simple and or cleavage polyembryony. Double-stained 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)