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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).

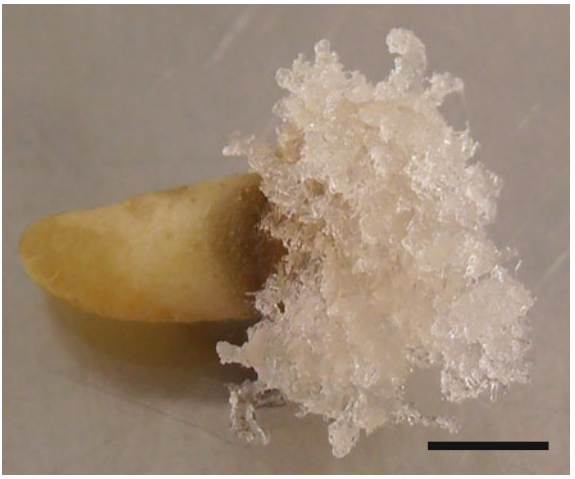

Fig. 1 Initiation of embryonal masses from *Pinus radiata* megagametophytes cultured at 23 °C on EDM (Walter et al. 2005), bar 0.2 cm

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.

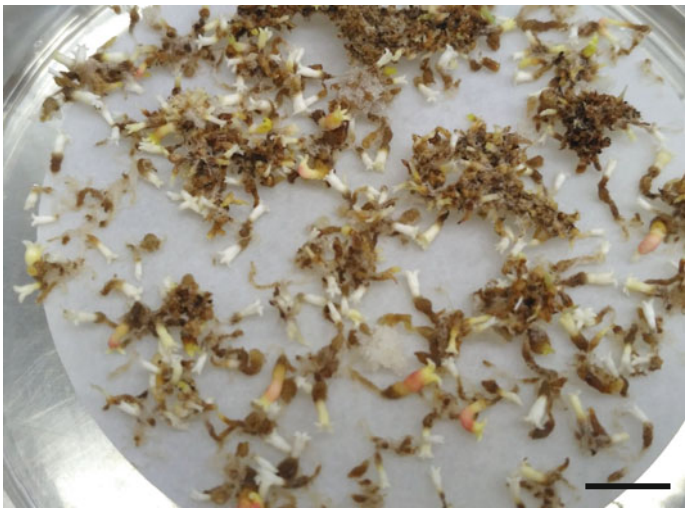

Fig. 2 Maturation of *Pinus radiata* somatic embryos cultured at 23 °C on EDM (Walter et al. 2005), bar 1.1 cm

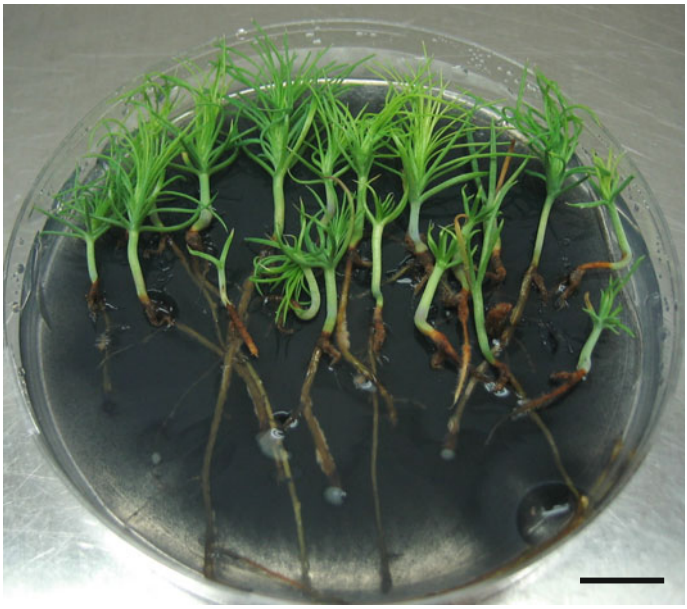

Fig. 3 Germination of *Pinus radiata* somatic embryos cultured at 23 °C on half-strength macronutrients LP (Quoirin and Lepoivre 1977, as modified by Aitken-Christie et al. 1988), bar 1.4 cm

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.

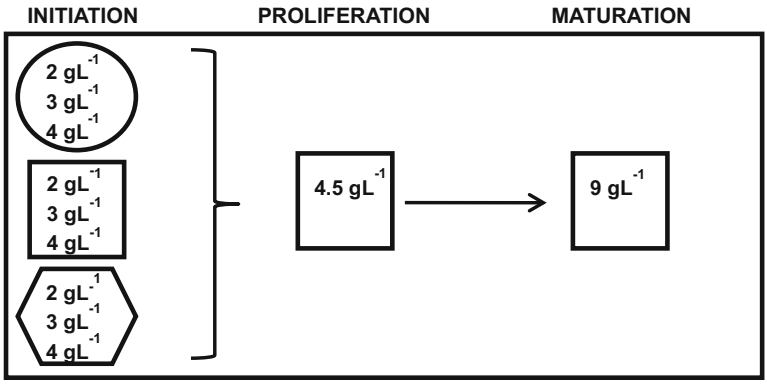

Fig. 4 Scheme of the experimental design, cultures were stored at initiation at three different temperatures: 18 °C (circle), 23 °C (square) or 28 °C (hexagon) and at three different agar concentrations (inside circles, squares and hexagons). The rest of the process was carried out under standard conditions

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

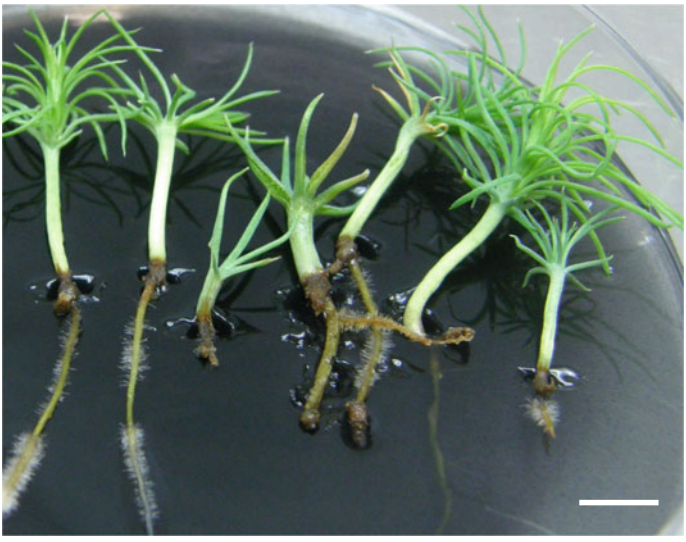

Fig. 6 Germination of *Pinus radiata* somatic embryos after 12 months of storage at 4 °C, bar 0.8 cm

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

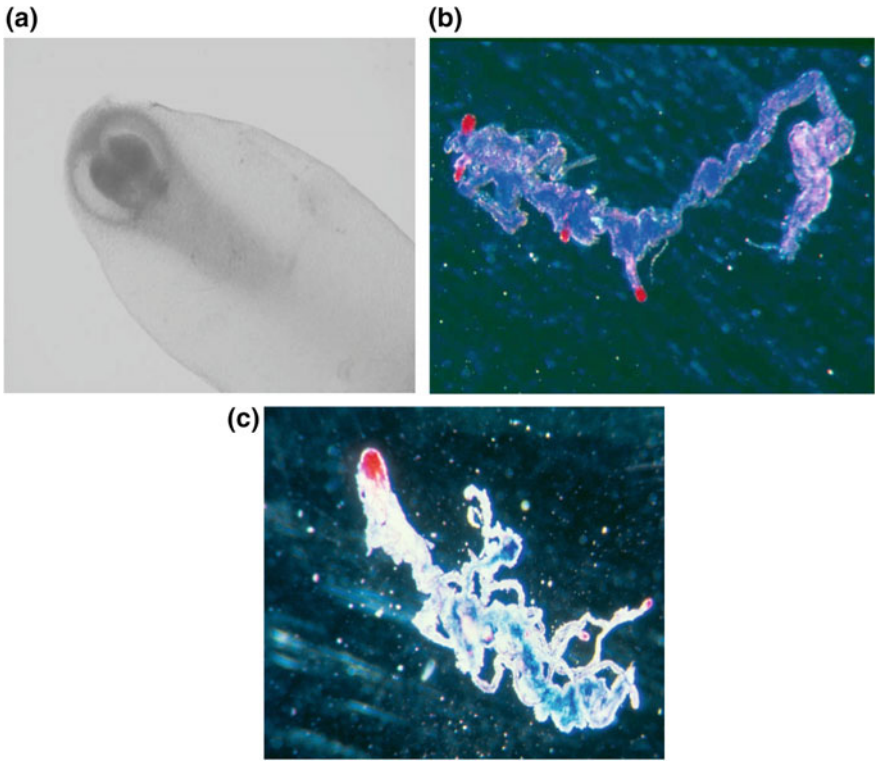

Fig. 1 Natural zygotic embryogenesis in *Pinus taeda*. **a** Megagametophyte with two archegonia visible shortly after fertilization. **b** Polyembryony several weeks after fertilization. Multiple early-stage zygotic embryos are visible resulting from simple and or cleavage polyembryony. Double-stained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

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

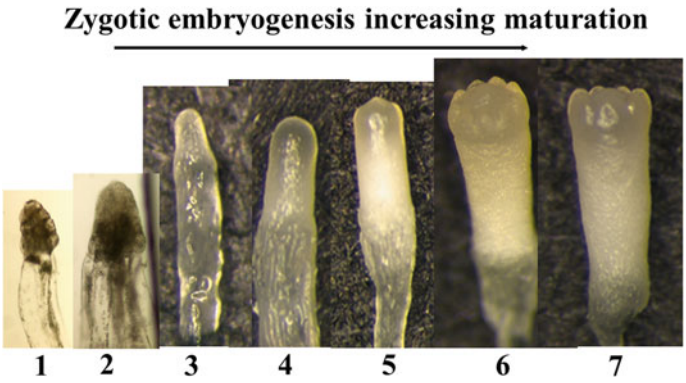

Fig. 2 Developmental stages for zygotic embryogenesis in loblolly pine. Based on Pullman and Webb (1994). Adapted from Cairney and Pullman (2007)

(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.

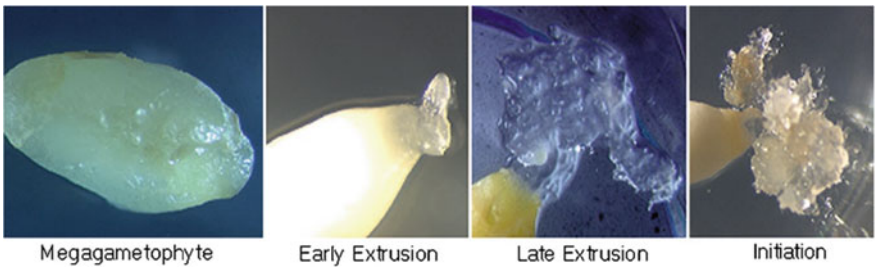

Fig. 3 Typical sequence of embryogenic tissue initiation in loblolly pine. Reproduced from Pullman et al. (2003d) with permission from Springer

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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Fraser Fir (*Abies fraseri* [Pursh] Poir.)

Gerald S. Pullman and John Frampton

1 Introduction

Fraser fir (*Abies fraseri* [Pursh] Poir.) is a coniferous species native to the Southern Appalachian Mountains of the Eastern United States and occurs in disjunct high-elevation populations in Western North Carolina, Eastern Tennessee, and Southwest Virginia. It is commercially grown as a popular Christmas tree for the dense and fragrant foliage and excellent post-harvest needle retention and is a multimillion-dollar business in the Southern Appalachian Mountains. In North Carolina alone, revenues of over $100 million occur annually (NC Coop. Ext. 2017).

The balsam woolly adelgid (*Adelges piceae* Ratzeburg), an introduced insect pest, and air pollution have killed most old growth Fraser fir. Prospects for young wild trees are uncertain (McManamay et al. 2011). The adelgid often kills trees within 2–7 years of infestation in natural and commercial stands. Global warming also poses a problem for wild and commercial populations. To survive, populations are expected to retreat to higher elevations and colder temperatures. According to the International Union for the Conservation of Nature and Natural Resources (IUCN), Fraser fir is vulnerable to extinction. As temperatures rise, it will become increasingly important to conserve rare and valuable germplasm to maintain biodiversity (Fenning et al. 2008).

Seed production often begins at about 15 years of age with poor early seed crops that generally occur every other year. A vegetative propagation system is needed to

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produce trees with desirable growth characteristics, disease tolerance, and pest tolerance. Grafting and rooted cutting systems have been researched but are not commercially used on a large scale due to expense, low multiplication and undesirable maturation effects including plagiotropic growth (Hinesley and Frampton 2002; Hibbert-Frey et al. 2010, 2011; Rosier et al. 2004, 2005). Somatic embryogenesis (SE) technology may fill the clonal propagation need by (1) shortening the time to produce desired planting stock, (2) allowing control of genetic variation, (3) permitting commercial production of hybrids, and (4) facilitating genetic engineering efforts to add or combine desirable traits. From a conservation point of view, SE technology will facilitate long-term safekeeping through cryopreservation and relocation of the species if needed.

Conifer SE proceeds through four steps: initiation, multiplication, maturation and germination plus cryopreservation when storage of cultures is desired (Pullman et al. 2003a). Somatic embryogenesis in *Abies* was first reported by Schuller et al. (1989) for silver fir [*Abies alba* (Mill.)]. Other *Abies* species and hybrids have been shown to produce embryogenic tissue (ET) and mature embryos that continue to develop (Nørgaard and Krogstrup 1995; Salajova et al. 1996; Jasik et al. 1999; Salaj et al. 2005; Vondrakova et al. 2011; Korecky and Vitamvas 2011; Vookova and Kormutak 2014). Unlike many conifers, ET for *Abies* species is most often induced on medium with only cytokinins as phytohormones. Rajbhandari and Stomp (1997) started ET from one of 44 Fraser fir families using precotyledonary embryos; however, the one initiation that developed did not continue to grow. Guevin and Kirby (1997) reported a maximum induction frequency of 3.5% using isolated full-term Fraser fir embryos. Kim et al. (2009) reported 4.7 and 2.2% initiation for two Fraser fir families and 0% for three more sources using whole female gametophytes (FG) containing immature embryos at a proembryo developmental stage.

Improved protocols for ET initiation, culture capture and growth, somatic embryo development and maturation and cryogenic storage are presented based on the findings of Pullman et al. (2016).

2 Initiation of Embryogenic Tissue

2.1 Cone and Embryo Stage Collection

Fraser fir cones from conservation or breeding programs are used to initiate an ET culture. Open or cross-pollinated cones are collected in late June when immature embryo stages range from 2 to 4 (Pullman and Webb 1994, Fig. 1). Cones are transported or shipped on ice, received within 24–48 h and processed immediately or stored in plastic bags at 4–5 °C for several weeks. Note: to maximize initiation seed cone collection should target embryos at stage 3 (Fig. 2). Full term seeds may be used for ET initiation however; initiation percentages are often lower.

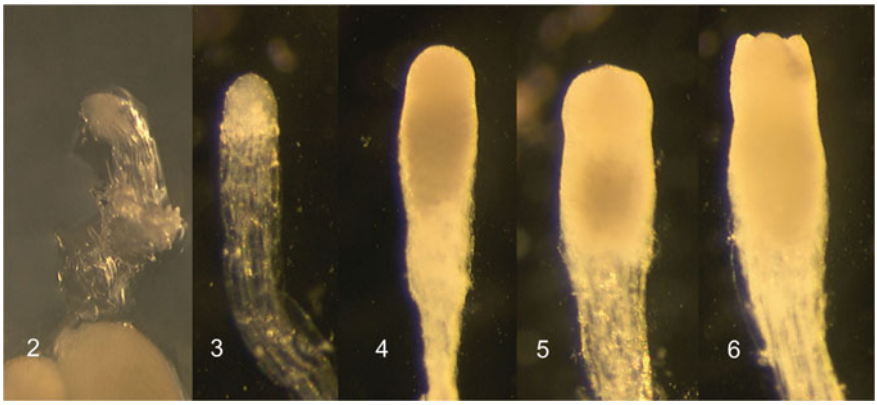

Fig. 1 Fraser fir zygotic embryos at stages 2–6 using the staging system of Pullman and Webb (1994). Adapted from Pullman et al. (2016)

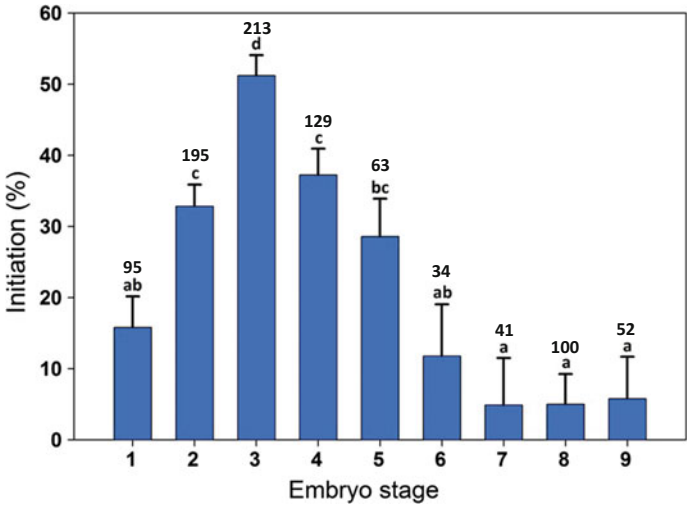

Fig. 2 Initiation percentages by embryo explant developmental stage using four families in 2009. Standard error bars are shown with a letter above the bar. Bars with the same letter are not statistically different by multiple range test at P = 0.05. The bar is based on the number of explants shown above the bar. Adapted from Pullman et al. (2016)

2.2 Initiation Medium Preparation

Medium FFI (Table 1) is prepared, and pH adjusted to 5.7 with KOH or HCl after addition of all ingredients except gelling agent and filter-sterilized materials. Medium is autoclaved at 121°C for 20 min. Filter-sterilized aqueous stock solutions of L-glutamine, brassinolide and ABA are added to medium cooled to about 55 °C.

Paclobutrazol (95.8% active ingredients, purchased from Duchefa, Netherlands) stock solution slurries containing 1 mg/mL are vortexed and rapidly added to the medium prior to autoclaving. At a concentration of 35 mg L^{-1}, paclobutrazol will dissolve fully. Currently we use brassinosteroids that can be purchased from Sigma-Aldrich (St. Louis, MO), Duchefa (Haarlem, The Netherlands) or PhytoTechnology Laboratories (Showcase Mission, KS). Brassinolide stock solutions are prepared in absolute ethanol (Aaper Alcohol and Chemical Co.). Thus, media with 0.1 μM brassinolide also contained 48 μL/L ethanol, a level that, in prior tests with other coniferous species, did not result in statistically significant changes in ET growth.

2.3 Seed Sterilization and Explant Preparation

Cones are opened and separated into individual ovuliferous scales. A small square is cut from the bract containing the undamaged seed attached to the scale. Damage may occur to the thin seed coat when it is detached from the ovuliferous scale due to overexposure to sterilization fluids. Seeds with ovuliferous scale or full term seeds are washed in running cold tap water for 10 min, agitated for 10 min in 10% Liqui-Nox (detergent) containing 2 mL Tween 20/L, and rinsed for 30 min with running tap water. Mature or immature seeds are sterilized with procedures developed for loblolly pine (*Pinus taeda* L.) (Pullman et al. 2005a, 2016). Seeds are agitated aseptically in 20% H_2O_2 for 10 min and rinsed five times for 5 min with sterile deionized water.

2.4 Aseptic Dissection

For immature seed, the coat, integuments and nucelli are carefully removed from the female gametophyte (FG). FG is carefully cut open and the dominant embryo lifted out while attached to the FG and the exposed embryo and FG are placed cut surface down onto initiation medium FFI (Table 1). For mature seed, coat, integuments and nucelli are removed, the FG is split and the mature embryo is removed and placed onto FFI medium without the FG present. Wu et al. (2012) has shown that loblolly pine full-term FG contain *myo*-inositol hexakisphosphate that inhibits early-stage somatic embryo growth. When Pullman et al. (2016) compared initiation for full term embryos with or without the FG present, more initiation occurred for explants without the FG. Explants can be placed on 7 mL of medium in 60 × 15 mm Petri plates or for cost savings, 7 mL of medium per wells of a multi-well plate such as Costar no. 3506 6-Well Culture Cluster Plates. Plates are wrapped in two layers of Parafilm and incubated at 23–25 °C in the dark.

Table 1 Fraser fir initiation, maintenance, germination and cryopreservation medium components

Components	Initiation	Capture	Maturation	Germination	Cryopreservation
mg/L	FFI	FFCP	FFM	397[b]	FFCR
KNO_3	–	–	–	1170	–
NH_4NO_3	–	–	–	206.3	–
K_2HPO_4	87.1	–	–	–	–
KH_2PO_4	–	340	340	85	340
$CaCl_2{\cdot}2H_2O$	–	–	–	220	–
$CaSO_4{\cdot}2H_2O$	37.8	37.8	37.8	–	37.8
$MgSO_4{\cdot}7H_2O$	394.2	394	394	185.5	394
KI	0.083	0.083	0.083	0.415	0.083
H_3BO_3	2.48	2.48	2.48	3.1	2.48
H_3PO_4	180.6	373	373	–	373
$MnSO_4{\cdot}H_2O$	18.6	18.6	18.6	8.45	18.6
$ZnSO_4{\cdot}7H_2O$	5.76	5.76	5.76	4.3	5.76
$Na_2MoO_4{\cdot}2H_2O$	0.103	0.103	0.103	0.125	0.103
$CuSO_4{\cdot}5H_2O$	3.75	3.75	3.75	0.25	3.75
$CoCl_2{\cdot}6H_2O$	0.012	0.012	0.012	0.125	0.012
$NiCl_2{\cdot}6H_2O$	1.188	1.188	1.188	–	1.188
$FeSO_4{\cdot}7H_2O$	16.68	16.8	16.8	13.93	16.8
Na_2EDTA	–	–	–	18.65	–
myo-Inositol	1000	1000	1000	100	1000
Sucrose	10,000	10,000	–	20,000	10,000
Maltose	–	–	40,000	–	–
Sorbitol	–	–	–	–	72,868
$C_{12}H_{10}Mg_3O_{14}{\cdot}9H_20$	266	266	266	–	266
L-Glutamine[a]	2000	2000	2000	–	2000
Thiamine·HCL	1	1	1	1.0	1
Pyridoxine·HCL	0.5	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5	0.5
Glycine	–	–	–	2.0	–
BAP	1.0	1.1	–	–	1.1
Brassinolide[a]	0.048	–	–	–	–
Paclobutrazol	0.33	–	–	–	–
Abscisic acid	1	–	5	–	–
$Na_2S_2O_3$	158.1	–	–	–	–
Acros PEG 8000	–	–	100,000	–	–
Activated charcoal	–	–	–	2500	–
Tissue culture agar	–	–	–	8000	–
Gelrite	3000	3000	3000	–	–
pH	5.7	5.7	5.7	5.7	5.7

[a]Filter-sterilized stock solution was added after autoclaving and cooling to 55–60 °C
[b]Medium 397 (Pullman et al. 2003a)

2.5 Embryogenic Tissue Evaluation

Initiation is evaluated 11–13 weeks after dissection. 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.

3 Embryogenic Tissue Capture and Maintenance

3.1 Embryogenic Tissue Capture

After 11–13 weeks ET from an initiation is transferred to capture medium FFCP (Table 1). Tissue weights are tracked over three three-week subcultures, and an initiation is considered "captured" when it reaches 200 mg. About half of the new initiations will continue growth to become captured. A target weight of 200 mg was selected based on observations in *P. taeda* where captured cultures reaching this mass tended to continue growth while cultures of less weight had a greater chance of growth decline (G. S. Pullman, personal observation). The remaining 50% do not grow even though ET formed during initiation or initially grew slowly and then stopped growth within several weeks to several months.

3.2 Embryogenic Tissue Maintenance

Once the culture is actively growing it can be maintained on gelled or liquid medium. Cultures grown on gelled medium (FFCP) are transferred to fresh medium after about three weeks with ET masses about 1 cm in diameter divided in two.

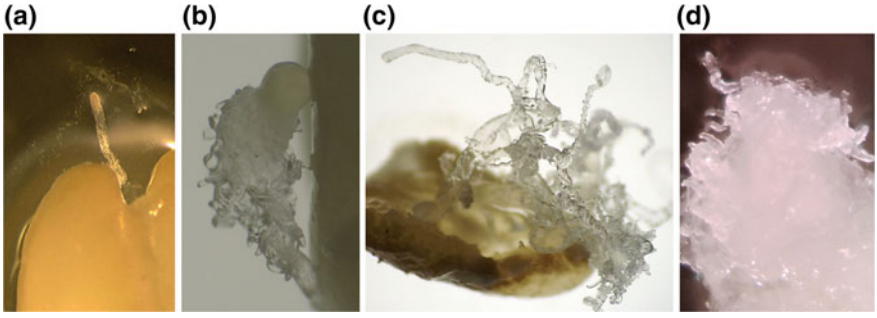

Fig. 3 Sequence of embryogenic tissue initiation in Fraser fir. **a** Female gametophyte and dominant zygotic embryo (stage 3) placed on medium. **b** Approximately one month after placement on initiation medium tissue from the dominant embryo is beginning to differentiate. **c** Embryogenic tissue is forming. **d** Proliferating embryogenic tissue

Observations suggest that tissue grown on gelled medium may produce slightly more advanced embryos during the development and maturation step possibly due to maintained pre-conditioning materials.

3.3 Embryogenic Tissue Suspensions

Cultures containing early-staged embryos grown in liquid medium (FFCP minus gelling agent) (Fig. 4) often grow faster, have less contamination, are more amenable to automated transfer, allow improved observation of embryos and have reduced labor requirements for maintenance (Pullman and Skryabina 2007).

To establish suspension cultures, pour 20 ml of FFCP liquid medium (i.e., without Gelrite) into a 125 mL Erlenmeyer flask under sterile conditions and inoculate with 1.0 g fresh weight ET. The ET should be harvested from the surface of cultures grown on gelled FFCP medium avoiding the wet compact tissue under the surface layer where embryo health declines. The harvested ET should consist of small tufts of embryos rather than large masses that may not break up in suspension. Culture growth rates vary by genotype but attempt to harvest tissue when the culture reaches its maximum growth rate, usually 14–21 days from the previous sub-culture. If ET is harvested too soon, the full multiplication potential of the culture is compromised. If harvested too late, the ET embryo quality may have declined. It is better to err toward harvesting too soon.

Place flasks containing inoculated liquid medium onto a gyratory (orbital) shaker set at 100 rpm in dark at 23–25 °C. Assess growth of the culture after 24 h by measuring settled cell volume (SCV). To measure SCV: (1) pour the flask contents into a 100 ml graduated cylinder under sterile conditions, (2) allow the ET to settle for 15 min, (3) measure the height (mm) of the 100 ml column with a ruler,

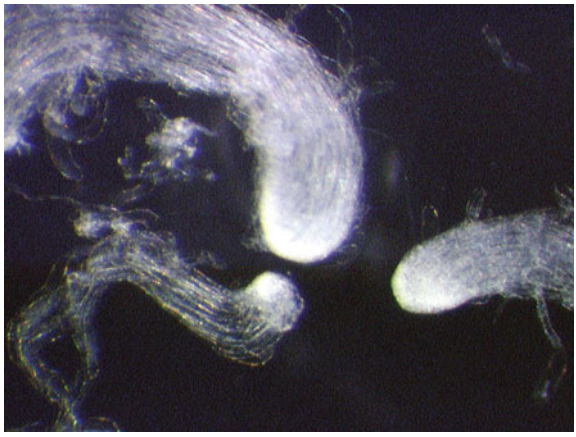

Fig. 4 Fraser fir somatic embryos grown in liquid culture suspension

(4) measure the height (mm) of the settled tissue, and (5) calculate SCV using the following equation, SCV (%) = [ET height (mm)]/[Total content height (mm)] 100. Expect an initial SCV of approximately 20%. Pour the cylinder contents back into the flask and return to the shaker. Reassess SCV weekly from the initial inoculation day. If no SCV increase is detected after 21 days, the attempt to establish a suspension culture has likely failed.

Once an ET culture reaches about 50% SCV, remove the liquid portion above the settled ET from the graduated cylinder using a sterile pipette. Pour fresh FFCP liquid medium into the cylinder bringing total volume to 40 ml (for a 250 ml flask) or 80 ml (for a 500 ml flask). Transfer into a larger flask and assess SCV weekly. The culture is considered established once ET achieves about 80% SCV. At this point, continue to multiply the culture by pouring 10 ml of swirled suspension into each for four new 250 ml flasks and bringing their total volume to 40 ml with fresh FFCP medium. For larger flasks, pour 20 ml of swirled suspension into each of four new 500 ml flasks and bring total volume to 80 ml.

In general, cultures are difficult to maintain long-term. Some culture contamination may occur and often cultures slowly decline over time. Signs of decline include slowing or lack of ET growth and/or loss of ability to develop cotyledonary embryos. To stop culture decline we store cultures cryogenically as soon after capture as possible. (See section on ET cryogenic storage.)

4 Somatic Embryo Development and Maturation

ET grown on gelled or liquid FFCP medium can be plated onto maturation medium. ET (pieces 5–8 mm) from gelled FFCP is placed onto FFM (Table 1). Alternately, ET suspensions grown in liquid FFCP can be settled, excess liquid removed, and aliquots of suspension (0.5–1.0 ml) dispersed onto support—material placed on FFM. Plates are wrapped in Parafilm and incubated in darkness at about 25 °C. After two 6-wk transfers to fresh FFM, somatic embryos are observed (Fig. 5).

In work with *Pinus taeda* embryo suspensions, Pullman and Bucalo (2011), used monofilament support spun from nylon fabric (06400JP-72 with 162 × 162 fibers/in and 60 μm openings) purchased from Decotex Inc., Pawling, New York. In tests, using 6, 29 and 15 genotypes, Pullman et al. (2016) found average yields of cotyledonary embryos of 17.2 per plate, 0.7 to 58.7/g of ET with an average across genotypes of 24.5 cotyledonary embryos/g and 22–925/g with an average of 227 cotyledonary embryos/g ET. Across the three tests, 46% of the genotypes produced cotyledonary embryos on FFM.

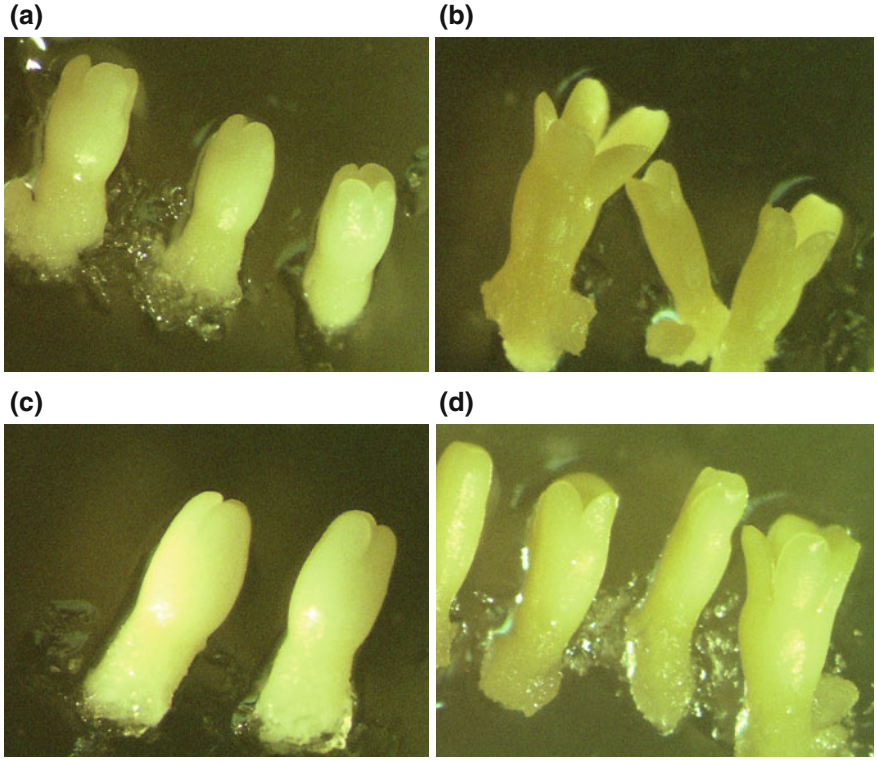

Fig. 5 Fraser fir cotyledonary embryos produced on maturation medium FFM. Four genotypes from two open-pollinated families are shown. Reproduced from Pullman et al. (2016) with permission from Springer

5 Somatic Embryo Germination

After three months on maturation medium, somatic embryos are selected that exhibit normal shape and maximal size. Embryos are placed horizontally on 20 mL of germination medium 397 (Table 1, Pullman et al. 2003a) in 100 × 20 mm Petri plates. Plates are wrapped with Parafilm and incubated at 25–26 °C for 7 days in the dark and then under lights with a 16/8 h (day/night) photoperiod from cool white fluorescent lamps at an intensity of ~30 mmol photons/m^{-2} s^{-1}. After 2 months, embryos are transferred to fresh medium and after eight weeks are observed for presence of roots and shoots. An embryo is considered germinated when it contains a root and a shoot. Within four weeks, hypocotyls expand followed by cotyledon expansion. At 4–8 weeks, apical primordia appear and slowly epicotyl and needles form and roots began to occasionally emerge (Fig. 6). Most germinated embryos grew slowly and set a tight apical bud that did not grow out. Two months of stratification on medium 397 at 4 °C increased germination

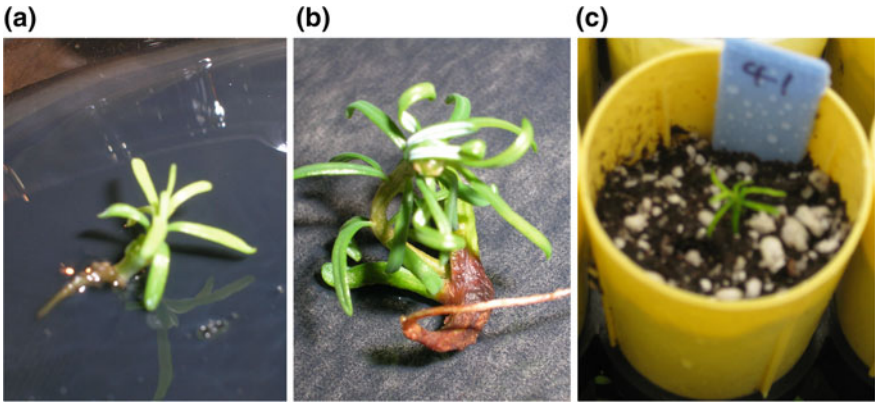

Fig. 6 Fraser fir somatic seedlings with root and epicotyl growth. Reproduced from Pullman et al. (2016) with permission from Springer

(Pullman et al. 2016), but somatic seedlings still grew slowly. Germination and seedling establishment is the weakest step in the Fraser fir protocol and requires further research.

6 Cryogenic Storage of Embryogenic Tissue

A modification of the method of Pullman et al. (2003a), described in detail by Pullman and Bucalo (2011) and Ma et al. (2012), can be used with ET grown in semisolid or liquid medium (Pullman et al. 2016). Cultures are grown on gelled or liquid FFCP medium (Table 2) with approximate monthly or bimonthly transfers, respectively. FFCR medium was developed from FFCP medium with added 0.4 M sorbitol. Cultures are incubated overnight on a shaker in liquid FFCR medium at a ratio of 1 g semisolid-grown tissue: 1.5 mL liquid medium or 1 part settled cells from liquid FFCR medium: 4 parts liquid FFCP medium. Filter-sterilized dimethyl sulfoxide (DMSO) is added slowly to cultures on ice to a final concentration of 5%. Then, 1.8 mL aliquots of the preparation are added to 2 mL Nalgene cryogenic vials. Vials are placed into a programmable freezer, cooled to −35 °C at a rate of 0.33 °C/min and then submerged in liquid nitrogen (LN). To retrieve cultures, vials are removed from LN, warmed in a 37 °C water bath for 1–2 min and contents are poured onto nylon mesh (Decotex Inc., Pawling, New York) resting on FFCP medium. After 1 h, and again 18 h later, the nylon mesh and cells are moved to fresh FFCP medium and maintained in the dark. After approximately six weeks viable tissue retrieved from LN will show signs of new ET growth. Once regrowth occurs, ET has been shown to grow normal cotyledonary embryos on FFM (Pullman et al. 2016).

7 Discussion

Protocols are presented that can multiply and preserve Fraser fir: ET initiation and capture, embryo maturation and germination and cryopreservation of cultures in LN. Results far exceed those previously reported for the species.

Initiation. Several medium additives and environmental conditions have been shown to improve ET initiation from conifers (Pullman and Bucalo 2011, 2014; Pullman et al. 2015). A blend of AL salts with brassinolide, paclobutrazol, ABA and sodium thiosulfate resulted in high-frequency initiation from immature or full-term Fraser fir embryos (Pullman et al. 2016). ET initiation averaged 6–62% for seed from 11 high-value mother trees. Isolated immature embryos showed initiation frequencies ranging from 18 to 50% on the best medium (FFI, Table 2, Pullman et al. 2016).

AL salts developed for subalpine fir based on metal analyses of seed tissues are high in P, Cu, and Ni and low in Ca compared to other salt bases used for conifer SE (Kvaalen et al. 2005). Unique to this medium is citrate in place of EDTA as an iron chelator. Citrate has been shown to be the major iron ligand in xylem sap, it provides energy as part of the tricarboxylate cycle and it stimulates early stage somatic embryo growth for loblolly pine (De Silva et al. 2008; Silva et al. 2009). Brassinosteroids improved initiation in loblolly pine, Norway spruce, Douglas fir (Pullman et al. 2003c), Himalayan blue pine (*Pinus wallichiana* A. B. Jacks., Malabadi and Nataraja 2007) and Fraser fir (Pullman et al. 2016). Paclobutrazol, a gibberellin synthesis inhibitor, promoted initiation and early-stage somatic embryo growth for several coniferous species (Pullman et al. 2005b). Loblolly pine, slash pine (*Pinus elliottii* Engelm.), Douglas fir and Norway spruce all responded to 1.0, 1.0, 1.0, and 0.33 mg L^{-1} paclobutrazol with increased initiation by 17, 48, 40, and 25%, respectively. Based on analyses showing ABA in early-stage FG, ABA increased loblolly pine ET initiation (Kapik et al. 1995, Handley 1997, 1999; Pullman et al. 2003b). Aitken-Christie and Parkes (1996) also found ABA increased initiation in Monterey pine (*Pinus radiata* D. Don) using dissected pre-cotyledonary bullet stage embryos and media without auxins or cytokinins. High initiation frequency for Douglas fir occurred when media contained ABA and decreased significantly when ABA was removed (Pullman et al. 2009). Sodium thiosulfate or other reducing agents have improved initiation in several coniferous species including Fraser fir (Pullman et al. 2016).

Initiation from immature conifer embryos depends on the embryo stage rather than time of year. Use of a staging system is critical to help understand variation in zygotic embryo development due to mother tree location, time of year and genotype (Fig. 1, Pullman and Webb 1994). ET grew from immature zygotic embryos at stages 1–6 with a clear optimum at stage 3 (Fig. 2). Cold storage (4°) can assist in obtaining optimal stages by dissecting seed sources with optimal stages first and allowing cones with early-stages to slowly advance towards the target stage while in storage. Exposed dominant embryos placed next to the gametophyte increased

initiation from immature seed when compared to leaving embryos in whole female gametophytes (Pullman et al. 2016).

Capture and maintenance. AL salts are the same as for initiation with the exception of potassium and phosphate which are increased 2.5 and 2.7 times. Brassinolide, paclobutrazol, ABA and sodium thiosulfate are removed and BAP is increased to 1.1 mg/L. Over six capture trials, Pullman et al. (2016) found about half of the initiations continued growth on FFCP medium. However, over time many cultures became contaminated, stopped growth, slowed growth or lost ability to produce cotyledonary embryos. To avoid significant culture decline, cultures were cryopreserved shortly after capture prior to the maturation step.

Significant culture decline has been reported for somatic embryogenesis in coniferous species (Nørgaard and Krogstrup 1995; Timmis 1998; Pullman et al. 2003b; Breton et al. 2006; Pullman and Bucalo 2014; Aurich et al. 2014). Recently, Aurich et al. (2014) reported that long-term culture of Caucasian fir [*Abies nordmanniana* (Steven) Spach] clones resulted in declining numbers of normally-developed mature embryos. Culture decline and lack of full embryo maturation are significant problems that impede commercial implementation of SE.

Maturation. FFM contains the same salts used for capture with the addition of 10% PEG 8000, 5 mg/L ABA and a change from 1% sucrose to 4% maltose. Pullman et al. (2016) reviewed development of this medium that is based on prior reports of *Abies* species maturation and experiences with embryo development for *Pinus taeda* and *Pseudotsuga menziesii*. In three tests, 23 of 50 genotypes produced cotyledonary embryos with yields ranging from 0.7 to 925/g ET.

Germination. Germination and conversion of *Abies* somatic embryos are often low and are reviewed by Pullman et al. (2016). Fraser fir had low germination for the few genotypes able to produce cotyledonary embryos. Partial drying did not improve germination, but two months of storage at 4 °C (stratification) did (Pullman et al. 2016). Long-term cold storage has been reported to improve germination and survival rates (Welty 2000; Liao and Juan 2015). Cotyledonary stage somatic *Abies* seedlings are known to go into dormancy under long day conditions and post acclimatization treatments at 5 °C or lower for at least six weeks will break this dormancy (Vookova and Kormutak 2014). Research on Fraser fir germination should focus on breaking dormancy induced during the conversion process. While our sequence of germination is slow and awkward compared to normal seed embryos, the ability to germinate somatic embryos from several genotypes produced on FFM medium provides a baseline for future improvements.

Cryopreservation. Cryostorage of seeds, somatic embryos or plant meristems is useful for storage of germplasm and can stop culture decline while in storage. Cryopreservation protocols based on slow-cooling and fast-thawing have been developed for Greek fir, Caucasian fir and hybrids of *A. cephalonica* and *A. alba* × *A. numidica* (Norgaard et al. 1993; Aronen et al. 1999; Misson et al. 2006; Salaj et al. 2010; Krajnakova et al. 2011). Early cryopreservation of Caucasian fir cultures stopped culture decline and allowed the production of stable quantities of somatic embryos after culture revival (Aurich et al. 2014). In our tests, 12 of 22 (55%) of the genotypes cryopreserved using FFCR medium survived, and

several genotypes were shown to produce cotyledonary embryos capable of germination after LN storage.

A highly effective initiation protocol is presented using dominant immature Fraser fir embryos. With efficient initiation we developed capture, maturation, germination and cryopreservation steps although more effort is needed. Our protocol may be useful to improve initiation in other recalcitrant firs and coniferous species.

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Bosnian Pine *Pinus heldreichii* Christ.

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1 Introduction

Bosnian pine (*Pinus heldreichii* Christ.) is a Tertiary relict subendemic to the mountains of Balkan Peninsula and a small area in southern Italy. It grows on Mediterranean and sub-Mediterranean-type mountains of Bosnia and Herzegovina, Montenegro, Serbia, Macedonia, Albania, Bulgaria, Greece and southern Italy, up to 2000 m above sea level, in areas where Mediterranean influence is either direct or indirect (Vidaković 1982). This pine mostly forms pure stands but may also constitute mixed stands with other conifers (Macedonian pine, Black pine, fir) and deciduous species (beech). Very often these stands form the upper forest line in mountains. In the past, Bosnian pine forests used to form a spacious and powerful forest belt in the Balkans, which through negative anthropogenic impact was reduced to a limited, small natural range of the present day that includes fragments of forests, sparse groves, small groups of trees or even individual trees (Janković 1991).

Bosnian pine generally grows on steep limestone slopes, in very poor arid soil, where there is no competition with species that require better soil and habitat conditions (Vukićević 1974). This pine is known for its wide ecological amplitude and its great adaptability to harsh conditions, which make its habitat requirements the smallest among the European tree species (Šumarska enciklopedija 1959). Its tolerance to low temperatures, extreme drought and polluted air, renders it a potentially important species for reforestation of areas devastated by erosion and flash floods, as well as of the urban environments (Jovanović 1971). Bosnian pine is

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a long-lived, slow-growing species reaching a height of up to 30 m, which is often used for ornamental purposes and in landscaping because of its pyramidal crown and low susceptibility to insect attacks.

In its natural habitats Bosnian pine regenerates by seeds. Seed production starts in trees over 40 years of age and varies greatly, not only from year to year, but also within a single tree (Đorđeva et al. 1972; Mičev 1972). Seeds of Bosnian pine are dormant, and in order to overcome the dormancy a chilling treatment of about six weeks at 3–5 °C is required (Stilinović 1985).

Problems encountered with traditional reproduction can be overcome by using the methods of tissue culture. A large number of high-quality plantlets can be obtained via zygotic embryo culture (Stojičić et al. 2008, 2012a). Successful plant regeneration employing embryogenic tissues as starting material has also been achieved by induction of adventitious buds (Stojičić et al. 1999, 2012b) and by micropropagation through axillary bud development (Stojičić and Budimir 2004). Besides organogenesis, somatic embryogenesis is another morphogenic process with a great potential for clonal propagation of Bosnian pine.

Somatic embryogenesis is the development of embryos from somatic, non-sexual cells. This is accomplished through a series of developmental stages, most of which are similar to those occurring during zygotic embryogenesis. As a method of conifer multiplication and propagation, somatic embryogenesis was first reported for *Picea abies* (Hakman and von Arnold 1985), and has later been attempted in a number of conifer species (Tautorus et al. 1991; Vujičić and Budimir 1995; Stasolla et al. 2002). In Bosnian pine, somatic embryogenesis was induced in culture of isolated megagametophytes (Stojičić et al. 2007). Conifers in general and especially genus *Pinus*, are considered recalcitrant for somatic embryogenesis (Stasolla et al. 2002). Ever since the first reports of somatic embryogenesis in conifers, the studies have been directed toward the improvement of the initiation frequency, proliferation rate, production of mature somatic embryos and embryo conversion into plantlets for many conifer species belonging to the genera *Pinus*, *Picea, Larix*, and *Abies*. However, for some species of these genera the embryo maturation and plantlet regeneration still remain just unfulfilled promises.

The protocol described here includes initiation, proliferation and maintenance of embryogenic tissue, as well as the initial stages of maturation of somatic embryos of Bosnian pine, which proved to be a limiting step in regeneration procedure.

2 Materials

2.1 Basic Material and Equipment

1. Immature cones of Bosnian pine at different developmental stages
2. Sodium hypochlorite, sterile distilled water, 250 ml Erlenmeyer flasks
3. Graduated cylinders, pipettes, sterile Petri dishes (glass and plastic, 90 mm), filter paper, parafilm

4. Strong forceps to open cones, stainless steel scalpels and forceps
5. Dissecting microscope, gas burners, laminar flow chamber
6. Refrigerator, autoclave, rotary shaker
7. Dark and lighted culture room at 25 ± 2 °C.

2.2 Culture Media

The basal medium (GD) is based on Gresshoff and Doy (1972) salts as modified by Sommer et al. (1975), supplemented with 3% (w/v) sucrose as a carbon source and solidified with 0.7% (w/v) agar. The medium is prepared from 4 stock solutions (macro and micro nutrients, iron chelate and vitamins) and organic additives as listed in Table 1, using ultrapure water (resistivity 18 MΩ cm).

Table 1 Basal GD medium composition

Constituents	Stock concentration (g L^{-1})	Final concentration (mg L^{-1})
Macro nutrients 10X stock, use 100 mL per L medium		
KNO_3	10.0	1000
$CaCl_2 \cdot 2H_2O$	1.5	150
$MgSO_4 \cdot 7H_2O$	2.5	250
$(NH_4)_2SO_4$	2	200
KCl	3	300
$NaH_2PO_4 \cdot H_2O$	0.9	90
Na_2HPO_4	0.3	30
Micro nutrients 100X stock, use 10 mL per L medium		
$MnSO_4 \cdot H_2O$	1.0	10.0
$ZnSO_4 \cdot 7H_2O$	0.54	5.4
H_3BO_3	0.3	3.0
KI	0.075	0.75
$Na_2MoO_4 \cdot 2H_2O$	0.025	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.25
$CoCl_2 \cdot 6H_2O$	0.025	0.25

(continued)

Table 1 (continued)

Constituents	Stock concentration (g L^{-1})	Final concentration (mg L^{-1})
Iron—EDTA 100X stock, use 10 mL per L medium		
$FeSO_4 \cdot 7H_2O$	2.78	27.8
$Na_2EDTA \cdot 2H_2O$	3.73	37.3
Vitamins 100X stock, use 10 mL per L medium		
Nicotinic acid	0.01	0.1
Pyridoxine HCl	0.01	0.1
Thiamine HCl	0.1	1
Organic additives		
Glutamine		50
Casein hydrolysate		500
Myo-Inositol		10
Sucrose		30,000
Agar		7000
Plant growth regulators		
2,4-dichlorophenoxy-acetic acid (2,4-D)	PGR stock solutions[a] 1 mg mL^{-1}	According to regeneration stage (Table 2)
N^6-benzyladenine (BA)		
Naphthalene acetic acid (NAA)		
Abscisic acid (ABA)		
p-Chlorophenoxyiso-butyric acid (PCIB)		

Macro and micro nutrients, iron chelate and vitamins stock solutions are kept at +4 °C until use
[a]Dissolve in 1 mL of absolute ethanol (2,4-D, ABA) or 1 N NaOH (BA, NAA, PCIB) and adjust volume to 10 mL with ultrapure water

A variation of GD medium, labeled GD1, where nitrogen salts were reduced to one half compared to the basal medium was also used successfully for embryogenic culture initiation.

Stock solutions of plant growth regulators (Table 1) are prepared beforehand, stored in refrigerator and added to the culture medium before autoclaving. Note that different stages of somatic embryogenesis described in this protocol differ in hormonal requirements as shown in Table 2.

The pH of the medium is adjusted to 5.8 with 1 N NaOH prior to autoclaving for 25 min at 115 °C and 1×10^5 Pa. The medium should be cooled down to 60 °C to minimize condensation, and dispensed into plastic Petri-dishes (25 mL per dish) in laminar flow chamber, sealed with parafilm and stored until use.

Table 2 Stages of Bosnian pine regeneration system, specific plant growth regulators and culture conditions, durations, and responses associated with each stage

Stage	Culture medium[a]	Medium additives (mg L^{-1})	Light regime[b]	Duration (days)
Embryogenic culture initiation				
Primary treatment	GD	2,4-D (2), BA (0.5)	Dark	5
	GD	NAA (2), BA (0.5)	Dark	5
	GD1	2,4-D (2), BA (0.5)	Dark	5
	GD1	NAA (2), BA (0.5)	Dark	5
Secondary treatment	GD	2,4-D (0.4), BA (0.1)	Dark	28
	GD	NAA (0.4), BA (0.1)	Dark	28
	GD1	2,4-D (0.4), BA (0.1)	Dark	28
	GD1	NAA (0.4), BA (0.1)	Dark	28
Embryogenic culture proliferation and maintenance				
	GD	2,4-D (0.2), BA (0.05)	Dark	28–56
	GD	NAA (0.2), BA (0.05)	Dark	28–56
	GD1	2,4-D (0.2), BA (0.05)	Dark	28–56
	GD1	NAA (0.2), BA (0.05)	Dark	28–56
Embryo maturation[c]				
	GD	PGR-free	Light	28
	GD	**ABA (0.3; 3; 6; 9; 12)**	Light	28
	GD	PGR-free + ABA (0.3-12)	Light	14 + 28
	GD	**Sucrose (5%), ABA (0.3–12)**	Light	28
	GD	Maltose (3%; 5%)	Light	28
	GD	PCIB (0.22)	Light	28
	GD	PCIB (0.22), ABA (6)	Light	28

Three replicates, each consisting of ten explants, were used per treatment and the experiments were repeated twice (n = 60). Total of 4080 seeds were used for embryogenic culture initiation experiments on GD media

[a]GD1 medium: nitrogen salts reduced to one half compared to the basal GD medium

[b]Incubation under 16-h photoperiod (45 μmol m^{-2} s^{-1}) or complete darkness at 25 ± 2 °C

[c]Treatments that initiated embryo maturation are marked in bold

3 Methods

3.1 Embryogenic Culture Initiation

Open-pollinated cones of *P. heldreichii* (Bosnian pine) were collected from large number of adult trees in natural stand located on Lovćen Mountain in Montenegro (Figs. 1 and 2), during the summer 2003, 2007, 2011 and 2013, and on Pešter plateau in Serbia, during the summer 2006, 2009 and 2010. Weekly collections of immature cones started in the last week of June and carried on over a period of four

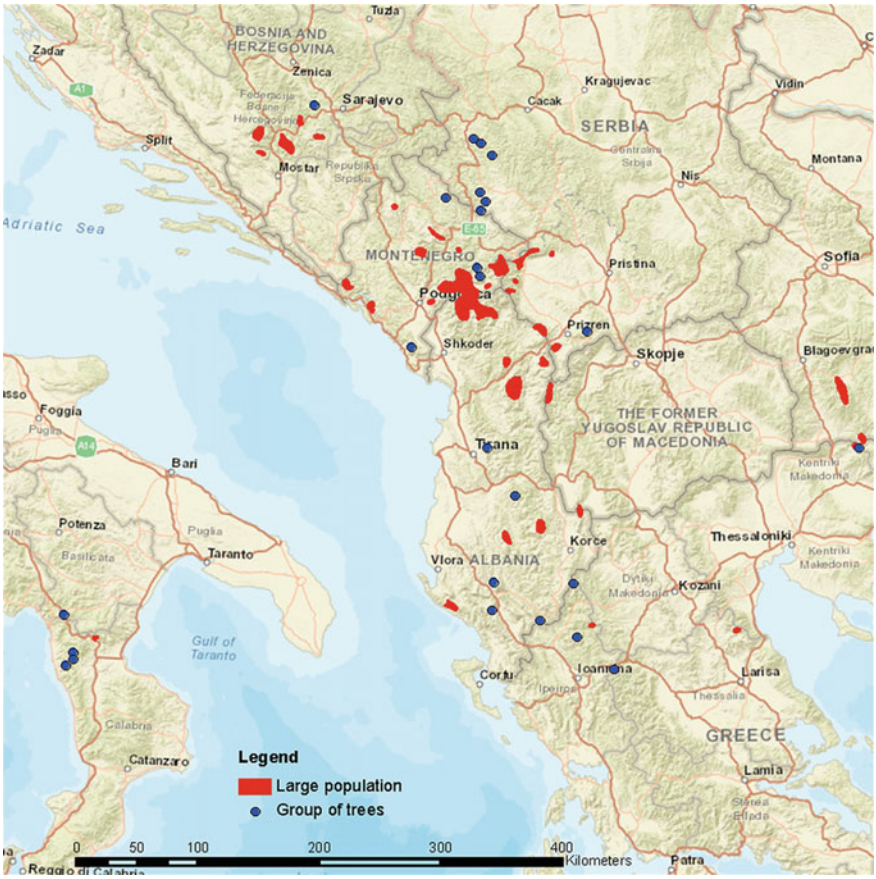

Fig. 1 Distribution map of Bosnian Pine (derived from Vidaković 1982; modified by Čokeša)

weeks (Table 3). Collected cones were stored in paper bags at 4 °C until use, for a maximum of 2 weeks.

1. For embryogenic culture initiation, remove the seeds from immature cones and wash for 24 h under running tap water.
2. Surface disinfect the seeds for 30 min in 20% (v/v) sodium hypochlorite, and then rinse with sterile distilled water three times in laminar flow chamber.
3. Transfer the sterile seeds to a sterile glass Petri dish.
4. Remove the seed coat under dissecting microscope using scalpel and forceps. Isolated megagametophyte appears translucent (cones collected in the last week of June and the first week of July) or slightly white (cones collected in the second and third week of July).
5. Place the excised megagametophyte (Fig. 3a) horizontally on the surface of the primary medium for embryogenic tissue initiation (Table 2).

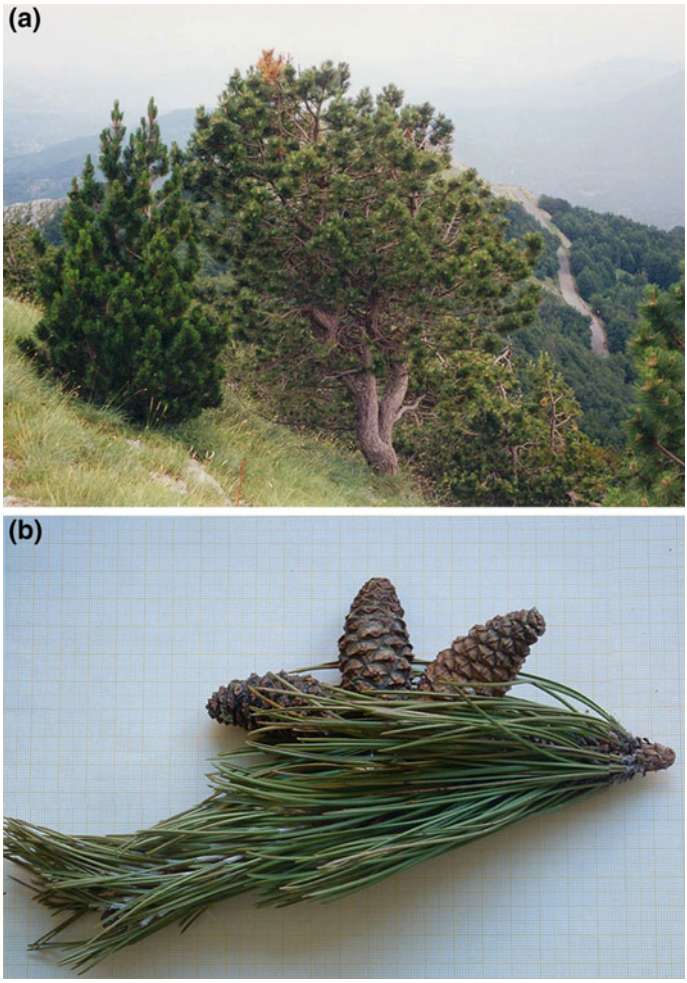

Fig. 2 **a** Bosnian pine in its natural habitat—Mt. Lovćen, Montenegro; **b** open-pollinated cones of Bosnian pine

6. Place maximum 10 explants per Petri dish, seal with parafilm and incubate in darkness for 5 days.
7. After the induction period (5 days), transfer the explants on secondary medium of the same composition but with the concentration of plant growth regulators decreased to one fifth of the initial value (Table 2). Transfer to fresh medium every 2 weeks and cultivate until the appearance of embryogenic tissue (28 days or longer). As long as the initial explant remains white, embryogenic tissue should not be detached, since its embryogenic potential can get lost.

Table 3 Effect of developmental stage of zygotic embryos of *Pinus heldreichii* on embryogenic tissue initiation percentage

Medium	Plant growth regulators (mg L^{-1})	Last week of June	1st week of July	2nd week of July	3rd week of July
		Developmental stage			
		Immature cleavage	Immature cleavage, precotyledonary	Precotyledonary, early cotyledonary	Early cotyledonary
		Initiation frequency (%)			
2003 (Lovćen)					
GD	2,4-D (2), BA (0.5)	6.7	–	–	–
GD	NAA (2), BA (0.5)	0	–	–	–
GD1	2,4-D (2), BA (0.5)	0	–	–	–
GD1	NAA (2), BA (0.5)	0	–	–	–
2006 (Pešter)					
GD	2,4-D (2), BA (0.5)	3.3	0	0	0
GD	NAA (2), BA (0.5)	6.7	0	0	0
GD1	2,4-D (2), BA (0.5)	16.7	6.7	0	0
GD1	NAA (2), BA (0.5)	3.3	0	0	0
2007 (Lovćen)					
GD	2,4-D (2), BA (0.5)	6.7	3.3	0	0
GD	NAA (2), BA (0.5)	3.3	0	0	0
GD1	2,4-D (2), BA (0.5)	10.0	3.3	3.3	0
GD1	NAA (2), BA (0.5)	6.7	0	0	0
2009 (Pešter)					
GD	2,4-D (2), BA (0.5)	6.7	3.3	–	–
GD	NAA (2), BA (0.5)	3.3	0	–	–

(continued)

Table 3 (continued)

Medium	Plant growth regulators (mg L^{-1})	Last week of June	1st week of July	2nd week of July	3rd week of July
		Developmental stage			
		Immature cleavage	Immature cleavage, precotyledonary	Precotyledonary, early cotyledonary	Early cotyledonary
		Initiation frequency (%)			
GD1	2,4-D (2), BA (0.5)	16.7	3.3	–	–
GD1	NAA (2), BA (0.5)	6.7	3.3	–	–
2010 (Pešter)					
GD	2,4-D (2), BA (0.5)	3.3	6.7	–	–
GD	NAA (2), BA (0.5)	6.7	0	–	–
GD1	2,4-D (2), BA (0.5)	10.0	0	–	–
GD1	NAA (2), BA (0.5)	3.3	1.7	–	–
2011[a] *(Lovćen)*					
GD	2,4-D (2), BA (0.5)	6.7	6.7	0	–
GD	NAA (2), BA (0.5)	3.3	0	0	–
GD1	PGR-free	3.3	0	0	–
GD1	2,4-D (2), BA (0.5)	13.3	3.3	0	–
GD1	NAA (2), BA (0.5)	6.7	3.3	0	–
2013 (Lovćen)					
GD	2,4-D (2), BA (0.5)	8.3	6.7	–	–
GD	NAA (2), BA (0.5)	6.7	0	–	–
GD1	2,4-D (2), BA (0.5)	8.3	0	–	–
GD1	NAA (2), BA (0.5)	1.7	1.7	–	–

For each collection date, induction period lasted 5 days

[a]Several modifications of basal GD medium, each supplemented with 2,4-D (2 mg L^{-1}) and BA (0.5 mg L^{-1}), were tested. Modified GD media were lacking either nitrogen, chloride, magnesium, dihydrogen or hydrogen phosphate. Initiation process completely failed when nitrogen, chloride or magnesium were omitted from the GD medium, or its frequency was very low (3.3%) when either dihydrogen or hydrogen phosphate were omitted from the GD medium

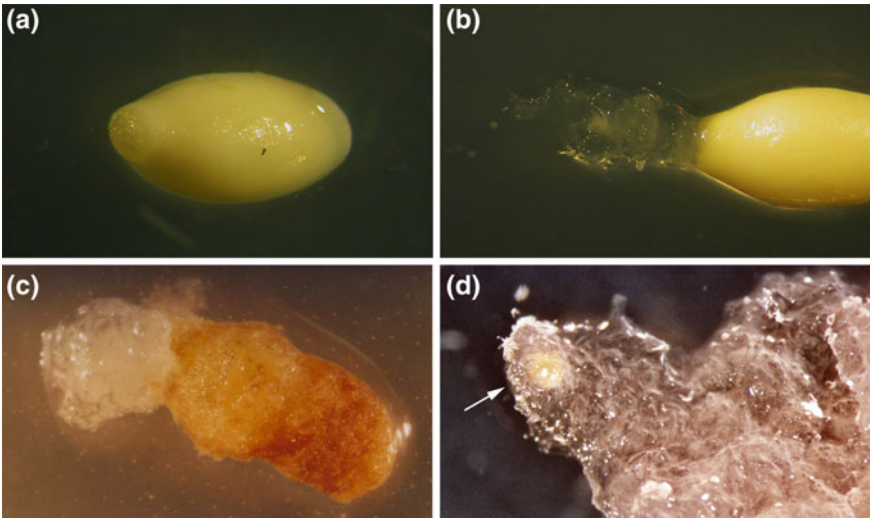

Fig. 3 Stages of Bosnian pine somatic embryogenesis. Embryogenic culture initiation: **a** Megagametophyte on induction medium; **b** extrusion of embryogenic tissue from the micropylar end. Translucent, glossy, mucilaginous tissue in the form of a strip spreads across the medium surface; **c** proliferation of embryogenic tissue mass; **d** initial stages of embryo maturation on medium containing ABA. *Note* embryo head (*arrow*) within mucilaginous matrix

After 4–5 weeks, immediately preceding the appearance of embryogenic tissue, the volume of initiated megagametophytes increases and white to translucent, glossy, mucilaginous embryogenic tissue protrudes from the micropylar end (Fig. 3b).

After the initial period characterized by slow growth, embryogenic tissue mass starts proliferating rapidly and reaches diameter of approximately 5 mm within a week. At this point, the embryogenic tissue should be separated from the megagametophyte which undergoes necrosis (Fig. 3c).

Screening of developmental stages of embryos from the seeds found in cones collected each week over a period of 4 weeks was performed in 2003.

Initiation frequency of embryogenic tissue formation was highest (up to 16.7%) when material collected in the last week of June was used, i.e. when initial megagametophytes mostly contained immature cleavage embryos (Table 3).

In cases when embryos in Bosnian pine megagametophytes were mostly at cotyledonary stage of development, the production of embryogenic callus was sporadic, regardless of the calendar year in which cones were collected. Cotyledonary embryos formed a non-embryogenic callus. At the beginning this callus resembled the embryogenic callus, but after proliferation it formed a loose tissue, where cells became separated from each other, elongated and vacuolated. This type of callus was not enveloped in mucilaginous gel, and the microscopic analysis of tissue failed to indicate the presence of any embryos.

The attempts to initiate embryogenic tissue in mature zygotic embryos isolated from mature seeds of Bosnian pine were unsuccessful. In addition to GD,

other basal media such as MS (Murashige and Skoog 1962) and LP (Von Arnold and Eriksson 1981) medium supplemented with different combinations of auxins (2,4-D or NAA) and cytokinin (BA) at various concentrations were also tested for induction of embryogenic cultures. Neither of these treatments yielded embryogenic response, since mature zygotic embryos have either germinated or formed a non-embryogenic callus (Stojičić et al. 2008).

Although developmental stage of the initial zygotic embryo was a decisive factor for embryogenic tissue initiation, its frequency also depended on the hormonal treatment and varied from year to year (Table 3).

3.2 Embryogenic Culture Proliferation and Maintenance

1. When embryogenic tissue mass reaches 5 mm in diameter, separate it from the megagametophyte and transfer to the medium of the same mineral composition but with concentration of the plant growth regulators decreased to one tenth of the initial value (Table 2). The embryogenic tissue is proliferated on this medium in darkness.
2. Transfer embryogenic tissue on fresh medium of the same composition every 2 weeks, when cultures reach diameter of 10 mm. Discard as much as possible of the colored and creamy white parts, mainly located at the flanks of the culture, transferring only white to translucent mucilaginous parts of the culture.
3. Growth pattern of different embryogenic lines ranges from very rapid growth (usually between 4th and 5th week of cultivation) to complete growth cessation. Some embryogenic cultures of Bosnian pine can be maintained in proliferating state for over a year.

3.3 Cytological Confirmation of Embryogenic Nature

The embryogenic nature of proliferated tissue is determined by staining with acetocarmine, followed by Evans blue.

1. Place a small piece of embryogenic tissue on a microscopic slide and submerge in a few drops of 2% (w/v) acetocarmine. Heat the slide for 10 to 15 s.
2. Wash with distilled water and stain with 0.05% (w/v) Evans blue for 5 to 10 s.
3. Wash with distilled water. Mount in water/glycerol mixture, place the coverslip and gently press to flatten the tissue. Seal with clear nail polish for examination under the microscope.
4. Embryogenic tissue contains cleavage embryos with embryonal head stained red and elongated suspensor stained blue (Fig. 4).

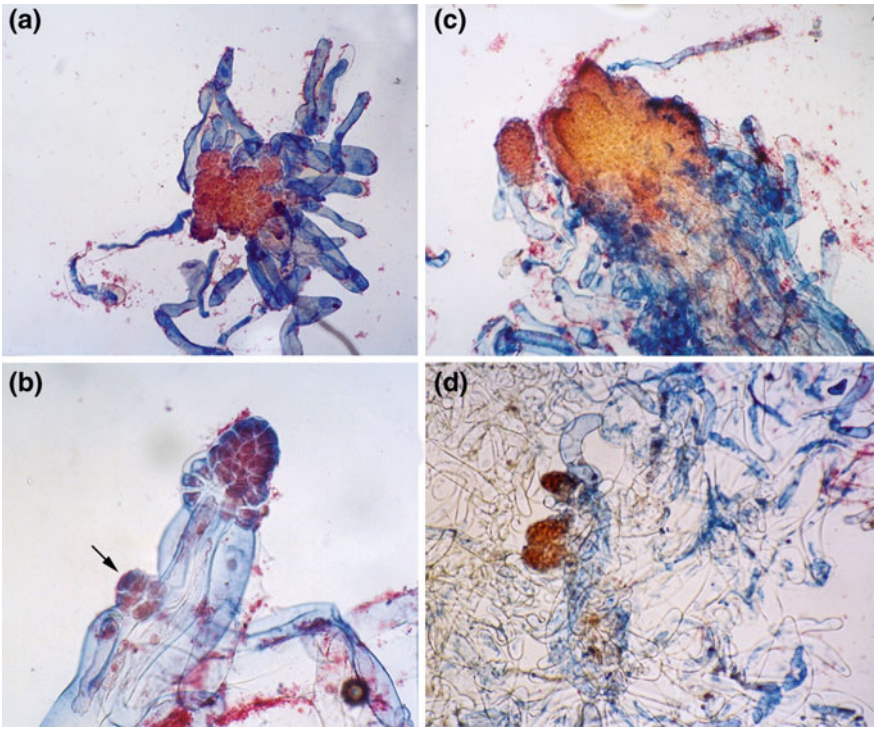

Fig. 4 Cytological aspects of proliferating Bosnian pine embryogenic tissue. **a** Somatic embryos with developing embryo head (*red*) and elongated suspensor (*blue*); **b** somatic embryo with a clearly differentiated embryo head, subtended by elongated suspensor. *Note* very early stage somatic embryo with a small, densely cytoplasmic head (*arrow*) and the emerging suspensor; **c** cleavage polyembryony; **d** somatic embryos within the network of senescing suspensor cells

3.4 Embryo Maturation

New embryos are constantly formed on culture medium containing 2,4-D or NAA and BA, but they fail to develop further and mature. For embryo maturation it is necessary to transfer the embryogenic tissue to a medium devoid of auxin and cytokinin, in order to inhibit cleavage polyembryony (Gupta 1995).

Presence of ABA is generally required for maturation of somatic embryos of conifer species (Stasolla et al. 2002). Cultivation of embryogenic tissue on medium containing ABA resulted in the initial stages of somatic embryo maturation, with highest embryo responsiveness achieved at 3 and 6 mg L^{-1} ABA and increased sucrose concentration (Table 2). Single embryos slightly increased in size and firm, opaque to yellowish embryo heads became noticeable within mucilaginous matrix (Fig. 3d). Tens of embryos were observed within one culture after one week. In the course of the next 2–3 weeks, embryogenic cultures became creamy yellow, while somatic embryos became brownish and failed to complete maturation process.

Different treatments tested for somatic embryo maturation are listed in Table 2. Supplementation of embryo maturation medium with maltose, antiauxin PCIB alone or in combination with ABA, or ABA following a 2—week PGR-free treatment did not induce maturation in Bosnian pine somatic embryos.

4 Research Prospects

Early steps of somatic embryogenesis in Bosnian pine are described in this protocol. Immature cleavage polyembryos were shown to be at the most suitable stage for the induction of embryogenic tissue, when cultured at high concentrations of auxin and cytokinin. However, further protocol modifications are needed in order to improve somatic embryo development and maturation. Future research should be aimed at a better understanding of the physiological regulation of the shift from proliferation to maturation. Investigations of the role of plant growth regulators in the process of Bosnian pine somatic embryogenesis should be accompanied by ultrastructural and biochemical studies.

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Stone Pine *Pinus Pinea* L.

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1 Introduction

Stone pine (*Pinus pinea* L.) is a native pine species of the Mediterranean ecosystem widely used due to its multiple applications. This species is characterized by a low genetic variation and high adaptive plasticity that allows a broad geographic distribution (Vendramin et al. 2008; Saéz-Laguna et al. 2014). Stone-pine dominated forests occupy about 0.75 million hectares from the Atlantic coast in Portugal to the shores of the Black Sea and the Mount Lebanon. In the last century, it has been clearly expanded in their natural southern and eastern Mediterranean region. In addition it has been introduced in South America, mainly in Chile, due to its economic relevance (Loewe et al. 2015). Multi-objective management is been employed in stone pine stands, using this species for purposes such as protection against soil erosion, ecosystem restoration and farmland afforestation. However the most important commercial application is the production of timber and pine nuts (Calama et al. 2008).

The Iberian Peninsula accounts for about 75% of the stone pine area, being Portugal the main pine nut producer followed by Spain (Mutke et al. 2013a). The stone pine nuts are ancient components of the Mediterranean diet because of their high nutritional value ascribed to high amounts of linoleic acid, becoming one of the most expensive dried fruit (Nergiz and Dönmez 2004). Recently a strong competition comes from pine nuts of other species, mostly *P. koraiensis* from China and *P. gerardiana* from Pakistan, which reach a lower price although they are of lower nutritional quality (Mutke et al. 2013b). The current state of the art in Mediterranean pine nut production in forests and orchards has been discussed in two international meetings on Mediterranean stone pine for agroforestry (Agropine

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2011, Agropine 2016) (Mutke et al. 2013c, 2016). These meetings presented the major problems that must be solved to increase the production in agroforestry systems, such as a more effective control of cone pests, the lack of traceability, pine nut labelling, and identification of the botanical species and the country of origin. In the last years a severe loss of seed-per-cone yield has been reported in all main producing countries, as consequence of global warming (Mutke et al. 2005) and the conifer seed bug *Leptoglossus occidentalis* (Strong 2006) among other factors. Furthermore, the annual variation of stone pine cone yield has been mainly related to weather factors (e.g. chilling, heat sums or water availability) and resource depletion (Mutke et al. 2005). Stone pine strobili are wind pollinated and need 3 years to ripen. Thus, the effect of environment factors during seed development on nut yield is dependant of annual variations. Since commercial cone production is still obtained from natural forests, the Spanish breeding program of stone pine is mainly focused on the improvement of cone production by planting selected materials in grafted orchards (Mutke et al. 2000).

Large-scale production of conifer trees through somatic embryogenesis (SE), combined with cryopreservation, has become a powerful biotechnological tool in tree improvement programs (Park 2014). A recent review has compiled the newest findings in conifers that affect the response of zygotic embryos to the SE induction, the long-term culture of early somatic embryos, the somatic embryo maturation and the growth of somatic plants in the field (Klimaszewska et al. 2016). For many *Pinus* species, initiation, maturation or plant regeneration efficiencies are still low and improvements in protocols are needed. The manipulation of the genetic mechanisms involved in embryo initiation, maturation and germination could help to overcome recalcitrance (Bonga et al. 2010). Furthermore, analytical studies of seed tissue, seed environment and gene expression in the megagametophyte and zygotic embryo would help to design more efficient protocols for SE (Pullman and Bucalo 2014). Since the usual explant to induce SE in *Pinus* species is the immature zygotic embryo enclosed in the megagametophyte, it has been suggested that the presence of the later could interfere the induction because it may produce toxic leachates and mediate the effect of the culture medium substances (Klimaszewska et al. 2016). The stone pine has one of the largest megagametophytes within conifers.

Plant regeneration by SE from immature zygotic embryos in stone pine using semisolid medium has been reported (Carneros et al. 2009), and the establishment of embryogenic suspension cultures for large scale production of somatic embryos is in progress (Celestino et al. 2012). As in other reports on protocol optimization to overcome low efficiencies at each stage of SE, improvements of maturation of stone pine somatic embryo have been achieved (Carneros et al. 2017a). Additional work dealing with the initiation of SE from mature zygotic embryos has also been reported (Celestino et al. 2015). Although embryogenic-like cultures were induced in both cotyledons and hypocotyls, the growth of the proembryogenic structures by continuous subcultures could not be maintained. It is reported that SE initiation ability decreases with zygotic embryo development in many conifers (Bonga et al. 2010). Research on initiation of SE from vegetative tissues of adult trees has also

been carried out (Carneros 2009). Slices from shoot-bud and expanding needles also produced cell masses of embryogenic appearance, but maintenance of these structures was not possible. These results were similar to the reported in other works on SE induction in tissues from mature conifer trees (Park et al. 2010; Humánez et al. 2012; Klimaszewska et al. 2016). A cryopreservation protocol of embryonal masses has been developed as a means to long-term store embryogenic lines (Carneros et al. 2017b). This chapter describes the protocols to regenerate stone pine plantlets by SE from immature zygotic embryos and the cryopreservation of embryogenic lines, focusing on the initiation and proliferation of embryogenic cell cultures, the production of somatic embryos and plant regeneration. Protocols used in the attempts to induce SE from vegetative tissues of adult trees are also described.

2 Somatic Embryogenesis from Immature Zygotic Embryos

2.1 Materials

1. Three-year-old immature green cones of stone pine collected from selected open-pollinated trees.
2. Commercial bleach (sodium hypochlorite: 3.5% active chlorine); ethanol (70%, v/v); Tween® 20 wetting agent and sterile distilled water.
3. Petri dishes (90 mm × 16 mm); vent culture box "Eco2" (E1654, Duchefa Biochimie B. V., 2003); Erlenmeyer flasks; airtight glass jars.
4. Pipettes; air-displacement piston pipettes; syringes; 0.22 μ solvent filter Millipore®. Clamp for opening the cones; stainless steel nut-cracker, forceps and scalpels. Storing paper bags; filter paper Whatman n° 2; filter-paper disks (80 g/m^2, 43–48 μm pore; Filter Lab, ANOIA; Barcelona, Spain).
5. Electronic balance; magnetic stirrer; pH-meter; refrigerator; autoclave; water bath with digital regulation; glass bead sterilizer; horizontal laminar-flow hood with ultraviolet light; horizontal orbital shaker (30 mm rotation radius); Büchner funnel with vacuum pump; dissecting stereo microscope Nikon SMZ; inverted stereo microscope Nikon TS100.
6. Plant growth regulators (PGR): 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP), abscisic acid (ABA; Sigma-Aldrich A1049).
7. Gelling agents; activated charcoal (AC, Sigma-Aldrich C6289); acetocarmine staining solution (1%, w/v); forest containers; peat and perlite; slow release fertilizer Osmocote®.
8. Mineral salts and organic components of culture media. Media composition is detailed in Table 1.
9. Tissue culture chambers and greenhouse.

Table 1 Medium composition for the different stages of somatic embryogenesis in *Pinus pinea*. All units are in mg/L

Constituent	M-mLV$_2$	M-mLV	UL-½mLV	ABA-mLV	AFC	SH	mLV-10G
NH_4NO_3	825	825	412	825	83	–	412
$NH_4H_2PO_4$	–	–	–	–	–	300	–
KNO_3	950	950	475	950	950	2500	475
$MgSO_4$ 7 H_2O	925	925	462	925	308	400	462
KH_2PO_4	170	170	85	170	340	–	85
$CaCl_2$ 2 H_2O	11	11	5.5	11	–	200	5.5
$Ca(H_2PO_4)_2$	–	–	–	–	200	–	–
$FeSO_4$ 7 H_2O	27.8	27.8	13.9	27.8	13.9	27.8	13.9
Na_2 EDTA 2 H_2O	37.3	37.3	18.7	37.3	18.7	37.3	18.7
H_3BO_3	31	31	15	31	15	6.2	15
$MnSO_4$ H_2O	21	21	10	21	10	16.9	10
$ZnSO_4$ 7 H_2O	43	43	21	43	21	8.6	21
Na_2MoO_4 2 H_2O	0.63	1.25	0.6	1.25	0.6	0.25	0.6
$CuSO_4$ 5 H_2O	0.25	0.5	0.25	0.5	0.25	0.025	0.25
$CoCl_2$ 6 H_2O	0.01	0.125	0.065	0.125	0.065	0.025	0.065
KI	2.1	4.15	2.1	4.15	2	0.83	2.1
$NiCl_2$	0.72	–	–	–	–	–	–
Nicotinic acid	0.5	0.5	0.25	0.5	0.5	0.5	0.25
Pyridoxine-HCl	0.1	0.1	0.05	0.1	0.1	0.5	0.05
Thiamine-HCl	0.1	0.1	0.05	0.1	0.1	0.1	0.05
Glicina	–	–	–	–	–	2	–
L-Glutamine	500	500	250	500	500	–	250
Myo-inositol	100	100	50	100	100	100	50
Casein hydrolysate	1000	1000	500	1000	1000	–	500
Sucrose	10000	20000	10000	60000	30000	30000	10000
Gerlite®	4000	4000	4 000	10000	–		10000
Plantagar	–	–	–	–	6000	6000	–
2,4-D (μM)	9	9.5	0.24	–	–	–	0.24
BAP (μM)	4.5	4.5	0.22	–	–	–	0.22
ABA (μM)	–	–	–	121	–	–	–

The pH of culture media is adjusted to 5.8 with 1 N KOH or 0.5 N HCl prior to autoclaving at 121 °C for 30 min. Solutions of L-glutamine and ABA are filter-sterilized and added to the cooling autoclaved medium in Erlenmeyer flasks, which are placed in a water bath set at 40 °C and stirred before pouring

2.2 Methods

Somatic embryogenesis comprises several steps: (1) embryogenic tissue initiation from immature zygotic embryos within megagametophytes, (2) maintenance and growth of embryogenic lines, (3) differentiation and maturation of somatic embryos, and (4) embryo germination and (5) acclimatization of somatic plants.

2.2.1 Embryogenic Culture Initiation

Use immature zygotic embryos at the cleavage polyembryony to pre-cotyledonary stage (the apical dome begins to develop) for initiation of embryogenic cultures. Female green cones are collected in a period between the middle of June to the middle of July.

1. Wash the cones with commercial liquid detergent under running tap water.
2. Submerge the cones in 70% (v/v) ethanol for 3 min, followed by immersion in a 10% (v/v) solution of commercial bleach for 10 min. Then, immerse in a fungicide solution (0.1% captan, 0.1% benomyl, w/v) for 1 min.
3. Air-dry the cones and store in paper bags at 4 °C for a maximum of 2 weeks while all seeds are dissected.
4. Remove the seeds from the cones by squeezing using a clamp (Fig. 1a).
5. Under laminar-flow hood, treat the seeds with 10% (v/v) plus 2 droplets of Tween®-20, for 15 min under continuous stirring, and rinse 3–4 times with sterile distilled water.
6. Remove the seed coats in a Petri dish containing sterile water, with nut-cracker, forcep and scalpel (Fig. 1b) and excise the female megagametophyte under stereomicroscope (Fig. 1c).
7. Place 10 megagametophytes with enclosed zygotic embryos horizontally in a 90 mm Petri dish containing 25 mL of M-mLV$_2$ induction medium, and seal with Parafilm®. This medium consist of a modified Litvay's medium (Litvay et al. 1985) with half-strength macroelements and full-strength microelements, Fe-EDTA and vitamins (Klimaszewska et al. 2001) but with microelements modified and supplemented with 9.5 μM 2,4-D, 4.5 μM BAP, 20 g/L sucrose and 4 g/L Gelrite® (G1910, Sigma-Aldrich Co., USA) (Table 1).
8. Incubate the cultures in darkness at 23 ± 1 °C over up to 12 weeks without subculture.
9. Check cultures initially every three days for the presence of eventual contamination. Transfer to fresh medium uncontaminated explants from contaminated cultures.
10. Between 4 and 12 weeks after plating on induction medium, embryogenic cell masses proliferate by cleavage polyembryony extruding from the micropylar end of the megagametophyte (Fig. 1d).

Fig. 1 Somatic embryogenesis in *Pinus pinea*. **a** Clamp to open cone by squeezing. **b** Removal of the sterile seed coat with nut-cracker. **c** Seed withdrawal with scalpel and forcep. **d** Extrusion of embryogenic tissue from megagametophyte containing a developing zygotic embryo. **e** Embryogenic tissue growing as clumps on maintenance medium (Petri dish, 90 mm Ø). **f** Embryogenic tissue growing as dispersed sample on maintenance medium (Petri dish, 90 mm Ø). **g** Cell suspension poured in a Büchner funnel and filtered under low pressure pulse. **h** Development of somatic embryos in ABA-containing medium (Petri dish, 90 mm Ø). **i** Maturation of isolated somatic embryos (bar = 1 mm). **j** Germination of somatic embryos in dishes that are tilted at an angle of 40° (Petri dish, 90 mm Ø). **k** Somatic plants growing in a vent culture box (bar = 15 mm). **l** Acclimatization of somatic plants growing in forest container in a humidifying chamber. **m** 15-month-old somatic plant grown in a greenhouse

2.2.2 Maintenance and Proliferation of Embryogenic Cultures

1. Once the proliferating cell mass reaches the size of about 10 mm in diameter, separate from the explant and transfer onto M-mLV maintenance medium (Table 1), subculturing biweekly onto fresh medium in darkness at 23 ± 1 °C. This medium consists of the modified Litvay's nutrient medium (Klimaszewska et al. 2001)
2. Subculture separately the embryogenic tissues from individual seeds.
3. For the maintenance of a vigorous proliferation, collect small pieces of tissue at the mass periphery and subculture onto fresh maintenance medium (7 individual clumps of about 100 mg of fresh weight (FW) per dish) (Fig. 1e).
4. For proliferation enhancement in those embryogenic lines showing low growth, suspend 100 mg of tissue collected as above in 10 mL of liquid maintenance medium, vigorously shake to break up the clump into a fine suspension, and pour onto a filter paper disk under low-pressure pulse to drain the liquid. Place the filter paper with attached cells onto semisolid maintenance medium and subculture biweekly (Fig. 1f).
5. Determine the growth rate of the embryogenic line as the relative fresh weight increase within 2-week intervals (final fresh weight minus initial fresh weight and divided by initial fresh weight).
6. To confirm the presence of early somatic embryos, collect small pieces from the embryogenic mass periphery and place on a glass slide with 1% acetocarmine (w/v) for 3–4 min, rinse gently with water and heat slightly for 5 s. The embryonal head cells (smaller cells with large nuclei and dense cytoplasm) stain bright red.

2.2.3 Embryo Maturation

1. The embryogenic tissue is preconditioned by subculturing small clumps on UL-½ mLV medium (Table 1) prior to maturation. This medium is made up of half-strength mLV medium with reduced concentrations of PGRs (0.24 μM 2,4D and 0.22 μM BAP), 10 g/L sucrose and 4 g/L Gelrite®.
2. Kept cultures in darkness at 23 ± 1 °C for one month with a subculture onto fresh medium after two weeks.
3. Collect pieces of preconditioned tissue (50 mg FW) from the periphery of the masses and suspend them in 5 mL of liquid ½ mLV medium lacking PGRs and glutamine, but containing 10 gl/L of AC. Pour the suspended tissue onto filter paper placed in a Büchner funnel, and apply a vacuum pulse to drain the liquid (Fig. 1g).
4. Place the filter paper disk with embryogenic tissues coated by AC particles into unsealed sterile empty Petri dish (60 mm diameter) for partial desiccation (about 23% water loss) under laminar hood flux for 6 h at room temperature.

5. Transfer the filter paper with the attached cells to a Petri dish (90 mm diameter) filled with 25 mL of ABA-mLV medium supplemented with 60 g/L sucrose and 10 g/L Gelrite® for maturation (Table 1).
6. Incubate the cultures in darkness at 23 ± 1 °C, for 4 month with monthly subcultures onto fresh medium (Fig. 1h).
7. Pick the immature somatic embryos (dome-shaped head region and with elongated suspensor cells) from proliferating tissues and place them in the same Petri dish but on the medium surface outside of the filter paper disk until completing their development (Fig. 1i).

2.2.4 Embryo Germination

1. Harvest mature somatic embryos (embryos with opaque body, well-defined cotyledons, embryonal root caps and smooth hypocotyls) and place horizontally onto AFC germination medium, all with the cotyledons facing in one direction. This medium consists of AFC nutrient medium (Bonga 2004) without PGR, supplemented with 30 g/L sucrose and 6 g/L Plantagar® (S1000, B&V, Italy).
2. Store embryos in darkness at 4 °C for 1 month.
3. Transfer the Petri dishes to a growth chamber at 23 ± 1 °C, with a 16-h photoperiod and photosynthetic photon flux density (PPFD) of 17–20 μmol m^{-2} s^{-1} provided by mixed Sylvania Gro-lux and Philips cool-white fluorescent tubes.
4. Place the Petri dishes tilted to a slanted position at an angle of approximately 40°, with embryos positioned with their embryonal root caps pointing downward (Fig. 1j).
5. Transplant aseptically those germinated somatic embryos with their root longer than 10 mm to vent culture boxes filled with 350 mL of SH medium (Schenk and Hildebrandt 1972) without PGR, supplemented with 30 g/L sucrose and 6 g/L Plantagar® (Table 1) for further growth (Fig. 1k).
6. Place the developing somatic plants in a growth chamber at 23 ± 1 °C, and a 16-h photoperiod of 120–180 μmol m^{-2} s^{-1} PPFD for 3–4 months.

2.2.5 Acclimatization

1. Transplant ex vitro the plants that maintain growth to 240 mL forest containers filled with substrate (peat: perlite, 3:1, v/v) and 3 g/L of the slow release fertilizer Osmocote®.
2. Cultivate the plants at 23 ± 1 °C under 80% relative humidity and a 16-h photoperiod of 120–180 μmol m^{-2} s^{-1} PPDF for 2–4 months (Fig. 1l).

3. Transplant active growing plants to 1000 mL forest containers filled with the same substrate and transfer them to a greenhouse irrigated by nebulization to complete their acclimatization (Fig. 1m).

3 Cryopreservation of Embryogenic Tissue

Somatic embryogenesis and cryopreservation of elite genotypes are emerging as a key component of advanced forestry programmes in conifers. Stone pine embryogenic lines derived from selected open-pollinated trees have been cryopreserved using a 0.4 M sorbitol-5% PSD (PEG-sucrose-DMSO) cryoprotectant solution recovering of up to five out of the six tested lines. The culture on medium with high gelling agent concentration before cryopreservation was essential for successful cryopreservation. Cryopreservation modified the maturation ability of some embryogenic lines, restoring maturation in three of them. A few plantlets were regenerated and are growing under greenhouse conditions (Carneros et al. 2017b).

3.1 Materials

1. Actively growing embryogenic lines.
2. Petri dishes (90 mm × 16 mm); Erlenmeyer flasks; pipettes, air-displacement piston pipettes; forceps; filter-paper disks; syringes, 0.22-μ filter Millipore®.
3. Electronic balance; magnetic stirrer; pH-meter; refrigerator; autoclave; Büchner funnel with vacuum pump; orbital shaker; water bath; glass bead sterilizer; ice-maker.
4. 50 mL flask (Duran® Group); 2-ml cryovials (Nunc CryoTubeTM Vials; Thermo Fisher Scientific, Waltham, MA, USA); Cryo 1 °C "Mr. Frosty" Freezing Container (Nalgene™) (C1562; Sigma-Aldrich, Saint Louis, MO, USA); crushed ice; ultra-freezer; cryostorage tank with rack to accommodate cryoboxes.
5. Goggles and cryo gloves; surgery masks.
6. Dimethyl sulphoxide (DMSO), polyethylene glycol (PEG 4000), sorbitol, activated charcoal.
7. Liquid and semisolid (M-mLV) re-growth media (Table 1).

3.2 Methods

All manipulations have to be performed in a horizontal flow hood using aseptic techniques and sterile materials. The method described below is divided into three steps: cryoprotection, freezing and culture thawing.

3.2.1 Cryoprotection

1. Culture proliferating embryogenic lines on M-mLV maintenance medium with biweekly subcultures onto fresh medium. Alternatively culture samples on a modified maintenance medium (mLV-10G) with reduced water availability and lower PGR concentration (Table 1) for 4 weeks with one biweekly subculture. Keep cultures at 23 ± 1 °C in darkness.
2. Collect the samples from tissue growing on the M-mLV medium (2.8 g FW of tissue) or on the mLV-10G medium (1 g FW of tissue).
3. Suspend the tissue samples in 7.3 mL of liquid M-mLV medium supplemented with filter-sterilized 0.4 M sorbitol in 50 mL flasks. Place the flask on an orbital shaker (120 rpm) for 16 h at 23 ± 1 °C in darkness.
4. Prepare an ice-cold filter-sterilized 0.4 M sorbitol-PSD cryoprotectant solution. PSD contains 16.7% (w/v) PEG 4000, 16.7% (w/v) sucrose, 16.7% (v/v) DMSO.
5. Place the flasks with the cell suspension on ice and add 3.2 mL of a cold filter-sterilized 0.4 M sorbitol-PSD cryoprotectant solution dropwise over a period of 2 min up to 5% PSD final concentration. Shake the flask continuously when adding the cryoprotectant solution. Keep the cell suspension on ice for 30 min to equilibrate.

3.2.2 Freezing

1. Dispense aseptically 1.5-mL aliquots (400 mg of tissue) of cold cell suspension into 2-mL cryovials. Keep the cryovials on ice for a few min before placing into freezing containers.
2. Place the cryovials in precooled (0 °C) Cryo 1 °C "Mr. Frosty" filled with 100% isopropyl alcohol that provides critical repeatable 1 °C/min cooling rate.
3. Place the freezing container at −80 °C for 2 h, during which a slow cooling of the cell suspension takes place.
4. Place the cryovials in cryoboxes and plunge the rack of cryoboxes in liquid nitrogen.

3.2.3 Thawing

1. Remove the cryovials from the cryotank and immediately plunge them in a warm sterile water bath at 40 °C for 3 min until complete thawing.
2. Bring the cryovials to the laminar flow hood and surface wash them with 70% ethanol.
3. Manipulate the cryovials aseptically. Pour the cell suspension onto a filter-paper disk placed on a stack (four pieces) of sterile blotting paper, and maintain under laminar flow hood for 1 min to drain the cryoprotectant solution.
4. Place the topmost filter-paper disk with the thawed tissue onto maintenance medium (Table 1) lacking PGRs supplemented with 2 g/L AC for 2 h, at 23 ± 1 °C in darkness.
5. Transfer the filter-paper disk onto standard maintenance medium for 24 h, at 23 ± 1 °C in darkness.
6. Transfer the filter-paper disk onto standard proliferation condition and subculture biweekly.
7. As soon as enough quantity of healthy tissue is obtained, recover the tissue and maintain as clumps on standard proliferation condition (M-mLV medium, 23 ± 1 °C in darkness, biweekly subcultures) without filter paper.

4 Attempt to Induce Somatic Embryogenesis in Tissues of Adult Trees

Clonal regeneration by SE is still difficult for many conifers and is often limited to the use of juvenile explants as immature zygotic embryos. The recalcitrance of explants from adult trees to SE induction has prevented the cloning of selected trees (Bonga et al. 2010). The choice of the most responsive tissues may give a solution to recalcitrance problems. The regeneration potential of some species can be enhanced by the use of tissue culture-derived material, such as plants that were regenerated in vitro (Klimaszewska et al. 2011). Although explants from juvenile plants of some species may show limited responsiveness to SE induction, this reactivity is usually lost with ageing of the donor tree. If phenotypically selected trees could be cloned the clonal tests would be more efficient, and from them the desired genotypes could be cloned without the need of previous cryopreservation at juvenile stage (Bonga 2016). Concerted effort by several research teams in Canada, France, Finland, Spain and New Zealand to induce SE in adult pine trees in species as *P. pinaster*, *P. sylvestris*, *P. radiata*, *P. patula*, *P. strobus* and *P. contorta* have been carried out (Trontin et al. 2016). This review describes the results of SE induction in primordial shoots explants but, up to date, with very limited success. Overall, some cell lines initiated in these species showed embryogenic-like characteristics and were capable to develop until the late stages of early embryogeny,

but were unable to multiplicate through cleavage polyembryony, except in the case of *P. sylvestris.*

Somatic embryogenesis induction in explants of adult stone pine trees have been also attempted using the protocols described for other pines. Cultures have been initiated in shoot-bud, single juvenile needles and expanding secondary needles in separate experiments started in several year of collection (Carneros 2009). Responsive tissues have been produced by the three types of explants on two tested medium. Although induction of embryogenic-like cultures has been obtained they could not be maintained with continued subculture. Such behaviour of induced cell masses has been often observed in similar explants of the above mentioned pine species, especially in Spanish populations of *P. pinaster*, using similar explant type and culture conditions (Humánez et al. 2012).

4.1 Materials

1. Branch segments with dormant shoot-buds (about 20 cm long and 1 cm diameter without needles), cut from lower branches of the crown of selected adult trees growing in a stone pine clonal bank.
2. Fungicide solutions of 1 g/L each of Captosan R® (8% carbendazim plus 40% captan) and Benoagrex® (50% benomyl); 2 ml/L of Previcur N® (60.5% propamocarb); 70% ethanol; solution of sodium hypoclorite with 0.35% active chlorine (Millipore Tablets®, 1.5 g active chlorine); Tween® 20; sterile distilled water; acetocarmine staining solution (1%, w/v).
3. Growth chamber with illumination provided by mixed Sylvania Gro-lux and Philips cool-white fluorescent tubes; perlite; plastic trays.
4. Electronic balance; magnetic stirrer, pH-meter; refrigerator; autoclave; Büchner funnel with vacuum pump; orbital shaker; water bath; glass bead sterilizer; horizontal laminar-flow hood; inverted stereomicroscope.
5. Forceps; scalpel; Petri dish (90 × 16 mm); Parafilm® (American Can Company, Greenwich, CT); storing paper bags; filter paper Whatman n° 2 and filter-paper disks (80 g/m^2, 43-48 μm pore; Filter Lab, ANOIA; Barcelona, Spain).
6. Two induction media are used in all types of explants (Table 2). One of them is the same induction medium as described in Table 1 but with higher PGR concentration (H-mLV$_2$). The other medium, labelled as PJ-I, consists of a modified 505 nutrient medium (Pullman and Johnson 2002). The maintenance medium (R-mLV$_2$ and PJ-M) are made up of the same composition as the initiation medium but with the PGR concentration reduced to one tenth (Table 2).

Table 2 Composition of the different medium used for the culture of different explants from *Pinus pinea* adult trees. All units are in mg/L

Constituent	Induction PJ-I	Maintenance PJ-M	Induction H-mLV$_2$	Maintenance R-mLV$_2$
NH_4NO_3	200	200	825	825
KNO_3	910	910	950	950
$MgSO_4$ 7 H_2O	246.5	246.5	925	925
KH_2PO_4	136	136	170	170
$MgCl_2$ 6 H_2O	101.7	101.7	–	–
Mg $(NO_3)_2$ 6 H_2O	256.5	256.5	–	–
Ca $(NO_3)_2$ 4H_2O	236.2	236.2	–	–
$CaCl_2$ 2 H_2O	–	–	11	11
$FeSO_4$ 7 H_2O	13.9	13.9	27.8	27.8
Na_2 EDTA 2 H_2O	18.6	18.6	37.3	37.3
H_3BO_3	15.5	15.5	31	31
$MnSO_4$ H_2O	10.5	10.5	21	21
$ZnSO_4$ 7 H_2O	14.7	14.7	43	43
Na_2MoO_4 2 H_2O	0.125	0.125	0.63	0.63
$CuSO_4$ 5 H_2O	0.173	0.173	0.25	0.25
$CoCl_2$ 6 H_2O	0.125	0.125	0.01	0.01
KI	4.15	4.15	2.1	2.1
$NiCl_2$	–	–	0.72	0.72
Nicotinic acid	0.5	0.5	0.5	0.5
Pyridoxine-HCl	0.5	0.5	0.5	0.5
Thiamine-HCl	1	1	1	1
Glicina	2	2	2	2
L-Glutamine	450	450	500	500
Myo-inositol	1000	1000	100	100
Casein hydrolysate	500	500	1000	1000
Sucrose	–	–	10000	10000
Maltose	15000	15000	–	–
Gerlite®	2500	2500	4000	4000
AC	50	50	–	–
2.4-D (μM)	–		15	1.5
NAA (μM)	10	1.0	–	–
BAP (μM)	4	0.4	4.5	0.45

4.2 Methods

1. Wash the branches under running tap water with a strong brush.
2. Submerge the branches in a fungicide solution of 1 g/L each of Captosan R® and Benoagrex® for 20 min. Then, wrap in wet paper, place in plastic bags and store for no more than 7 days at 4 °C until used.
3. To promote shoot-bud swelling, secondary needles sprouting and brachyblast development (shoot derived from needle fascicles), force the branches under growth chamber condition. Insert the branches upright into wet perlite in plastic trays, and place in growth chamber at 25 ± 1 °C and 80–95% relative humidity under a 16-h photoperiod (PPFD, 90–100 μmol m^{-2} s^{-1}) (Fig. 2a).
4. Spray branches with a fungicide solution of 2 ml/L of Previcur N® before explant collection.
5. Excise swelling shoot-buds (1–2 cm of length) from branches after 2–3 weeks under high humidity conditions (Fig. 2b).
6. Wash under running tap water for 30 min, then surface-disinfect by shaking in 70% ethanol for 30 s, followed by shaking in a solution of sodium hypoclorite (0.35% active chlorine) plus two drops of Tween® 20 for 15 min. Shake vigorously and apply low-pressure pulse for removing bubbles. Wash in three times with sterile distilled water in the laminar-flow hood.
7. Remove the shoot-buds scales using sterile forceps (Fig. 2c). Disinfect the shoot-buds by shaking in 70% ethanol for 30 s, followed by shaking in the mentioned fungicide solution (1 g/L de Captosan R® and 1 g/L de Benoagrex®) for 10 min, followed by shaking in the sodium hypoclorite solution (0.35% active chlorine) plus two drops of Tween® 20 for 10 min, and three rinses in sterile distilled water.
8. Cut shoot-buds transversely into thin slices (0.5–1 mm) using sterile scalpel and stereomicroscope, from the shoot apex to the basal region. Then, arrange the slices on induction media according to their original position in the shoot-bud (Fig. 2d)
9. Excise developing brachyblast primordia (6–8 mm of length) from de middle region of branches after 3–9 weeks under high humidity conditions (Fig. 2e).
10. Disinfect entire brachiblasts by shaking in 70% etanol for 30 s, followed by shaking in the above mentioned fungicide solution (1 g/L de Captosan R® and 1 g/L de Benoagrex®) for 10 min, followed by immersion in the sodium hypoclorite solution (0.35% active chlorine) plus two drops of Tween® 20 for 15 min. Shake vigorously and apply low-pressure pulse to remove the bubbles. Wash in three times with sterile distilled water in the laminar-flow hood (Fig. 2f).
11. Remove the single juvenile needles sited at the brachyblast periphery using sterile scalpel and transfer to induction medium.

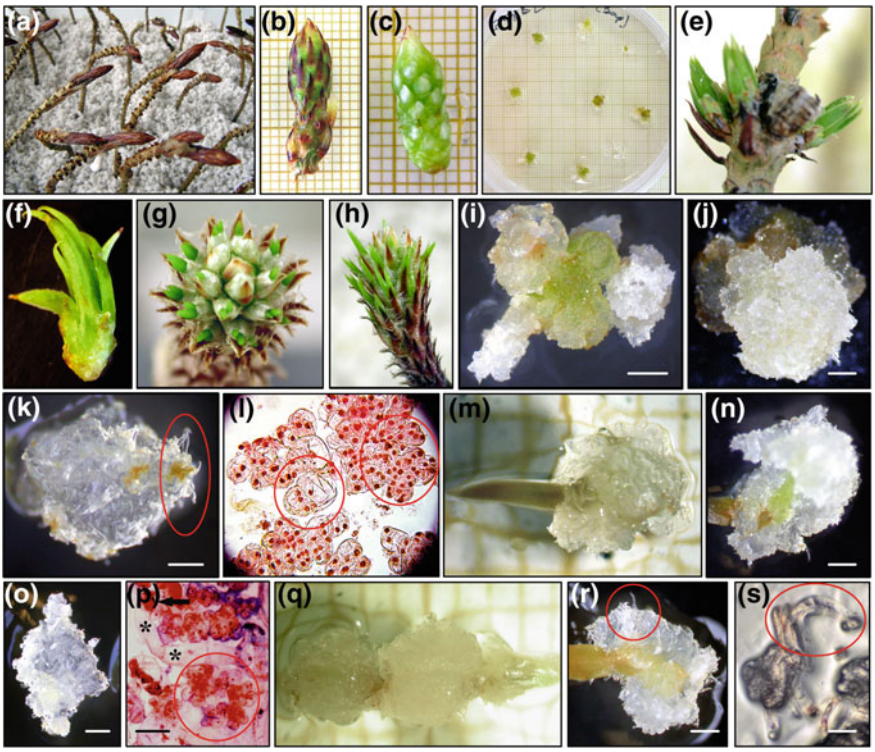

Fig. 2 Embryogenic-like tissues induced in explants from adult *Pinus pinea* trees. **a** Forcing of dormant shoot-bud growth under growth-chamber conditions. **b** Swelling of a shoot-bud. **c** Shoot-bud without scales. **d** Transverse shoot-bud slices on induction medium. **e** Developing brachyblast primordia from the middle region of branches. **f** Excised brachyblast with elongating needles. **g** Early stage of primordial secondary needle elongation. **h** Expanding needles through bud-scales. **i** Translucent tissue protruding from slices on H-mLV_2 initiation medium, after 3 weeks (bar = 2 mm). **j** Embryogenic-like tissue from slices, after 8 weeks (bar = 1 mm). **k** Filamentous proliferating cells on maintenance medium (circle) (bar = 0.5 mm). **l** Acetocarmine-stained cells mass showing clumps of ovoid cells with conspicuous nucleus and a proembryo showing an epidermal layer (circles) (bar = 100 μm). **m** Translucent cell mass raised from single juvenile needle cultured on PJ-I induction medium, after 2 weeks. **n** Mucilaginous proliferating cell mass from single juvenile needle, after 4 weeks (bar = 0.5 mm). **o** Proliferating embryogenic-like tissue on maintenance medium after 8 weeks (bar = 0.5 mm). **p** Acetocarmine-stained cells mass consisting of clump densely cytoplasmic cells (circle) and a initial stage of embryonal structure (arrow) with suspensor cell (stars) (bar = 100 μm). **q** Translucent tissue protruding around expanding secondary needle on PJ-I initiation medium, after 2 weeks. **r** Embryogenic-like tissue after 4 weeks on initiation medium, showing filamentous proliferating cells (circle) (bar = 0.5 mm). **s** Proembryogenic structure from embryogenic-like tissue (circle) (bar = 0.25 mm)

12. Excise the expanding secondary needles (8–13 mm of length) that have broken (Fig. 2g) and elongated through the bud-scales, after 4–6 weeks (Fig. 2h). Then, disinfect by shaking in 70% ethanol for 30 s, followed by shaking in a

solution of sodium hypoclorite (0.35% active chlorine) plus two drops of Tween® 20 for 5 min. Shake vigorously and apply low-pressure pulse to remove bubbles. Wash in three times with sterile distilled water in the laminar-flow hood.

4.2.1 Embryogenic-like Culture Initiation

1. Place slices of shoot-buds, single juvenile needles and expanding secondary needles horizontally on 90 × 16 mm Petri dish containing 25 mL of initiation medium (H-mLV$_2$ or PJ-I), and seal with Parafilm®.
2. Incubate the cultures in darkness at 23 ± 1 °C, over up to 8 weeks.
3. Between 3 and 4 weeks after plating on both induction medium, the shoot-buds generate mucilaginous and translucent tissue, located in the scale edges (needle fascicles) and cortical zone (Fig. 2i).
4. Between 2 and 3 weeks after plating the single juvenile needles generate a mucilaginous and translucent tissue, located on the cut surfaces (Fig. 2m). Filamentous cell masses proliferated on initiation medium after 4 weeks (Fig. 2n)
5. Between 2 and 3 week after plating, the expanding secondary needles produce a mucilaginous and translucent tissue, located on the cut surfaces or along the needles (Fig. 2q). Filamentous cell masses proliferated on initiation medium after 4 weeks (Fig. 2r).

4.2.2 Maintenance and Proliferation of Embryogenic-like Cultures

1. Separate the embryogenic-like tissue from the shoot-buds slices and subculture biweekly onto the induction medium (Fig. 2j). After 8 weeks in culture, subculture biweekly portions of the proliferative tissue onto maintenance medium (R-mLV$_2$, PJ-M) (Fig. 2k).
2. After 8 weeks on induction medium, subculture biweekly the embryogenic-like tissues from single juvenile needles (Fig. 2o) and the embryogenic-like tissues from expanding secondary needles onto maintenance medium. Proembryogenic structures were observed with an inverted stereomicroscope (Fig. 2s).
3. To confirm embryogenic nature, stain small samples from proliferating tissue with 1% acetocarmine, and examine microscopically to determine the presence of early somatic embryos.

Acetocarmine-stained cell masses obtained from the shoot-buds slices revealed clumps of ovoid cells with conspicuous nucleus, and proembryos showing epidermal layer (Fig. 2l). The cell masses formed from single juvenile needles showed

clumps of densely cytoplasmic cells and embryonal structures at initial stage of development (Fig. 2p).

5 Conclusion

Progress of plant regeneration in *Pinus pinea* by SE is limited to the cloning of immature zygotic embryos, as for many other *Pinus* species. Furthermore, SE response is restricted to only a few genotypes which limits the selection possibilities. This biotechnology needs further improvement to overcome the recalcitrance of this species, because induction and conversion rates are too low to be of commercial use. It would be advisable to test the effect of megagametophyte in the induction response of immature zygotic embryos, as well as the possible relationship between the seed environment (either abiotic or biotic defence factors during seed development) and the gene expression in megagametophyte and zygotic embryo of seed tissue.

In general, the responses to SE induction in tissues from adult trees were similar among pine species and, to date, produced very limited success. The initial embryogenic-like tissue failed to grow or displayed reduced growth and changed appearance with respect to true embryonal-suspensor cell masses after a few subcultures. However the reported observations are encouraging. Overall, there is no doubt that there are challenges in regeneration by SE in zygotic embryo and tissues from adult trees of stone pine, as it happens in other *Pinus* spp.

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Somatic Embryogenesis of Brazilian Conifer *Podocarpus lambertii* Klotzsch ex Endl.

Miguel Pedro Guerra, Leila do Nascimento Vieira and Hugo Pacheco de Freitas Fraga

1 Introduction

Podocarpus (Podocarpaceae) is the second largest genus among conifers, with 97 species mainly distributed in the Southern Hemisphere (Farjon and Filer 2013). *P. lambertii* Klotzsch ex Endl. is indigenous to one of the 25 biodiversity hotspots of the world, the subtropical moist forest ecoregion of the Atlantic Forest Biome (Myers et al. 2000; Farjon and Filer 2013). Considering that *P. lambertii* conservation status is classified as near threatened (IUCN 2014), it is at least desirable the development and use of biotechnological tools, such as somatic embryogenesis (SE), with potential for clonal propagation and ex situ conservation of endangered plant species.

The SE process involves reprogramming of somatic cells toward the embryogenic pathway. Thus, a somatic cell dedifferentiates and subsequently redifferentiates, resulting in the production of a mature embryo and further a whole plant. Besides the potential for conservation purposes, these features make SE a suitable model system for the study of morphophysiological and biochemical aspects during the early development in higher plants (Fehér et al. 2003).

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In conifers, plant regeneration via SE is achieved after a series of distinct steps: induction, establishment and multiplication of proembryogenic masses (PEM), maturation, and plant regeneration (Von Arnold et al. 2002). Protocols for plant regeneration based on SE have been established for many conifers species, but mostly for members of family Pinaceae (Tautorus et al. 1991; Filonova et al. 2000; Klimaszewska et al. 2007; Li et al. 2008).

Species from the closely related families Araucariaceae and Podocarpaceae are in general considered recalcitrant to SE. In *Araucaria angustifolia* (Araucariaceae), SE induction is limited to globular-staged zygotic embryos, and differently from most other conifer species, both induction and multiplication of PEM can be carried out in culture medium with plant growth regulators (PGR) supplementation or PGR-free (dos Santos et al. 2002; Fraga et al. 2015; Guerra et al. 2016). However, the transfer of PEM to a maturation phase I (PGR-free) culture medium with a high osmotic potential is essential to promote polarization (Fraga et al. 2015). The presence of polarized proembryos in the PEM of *A. angustifolia* is essential for maturation in culture medium supplemented with abscisic acid (ABA) and osmotic agents (Fraga et al. 2015).

Somatic embryogenesis is affected by many factors, such as genotype, explant, PGR, osmotic agents, redox state, and light. A thiol tripeptide formed by glutamic acid, cysteine and glycine (-glu-Cys-gly), the glutathione (GSH), has been show to improve the initial somatic embryo development, as it provides a reducing environment (Vieira et al. 2012; Fraga et al. 2016), while a shift to an oxidizing environment is required for late-stage development (Stasolla 2010). The influence of GSH on cell division and differentiation has been reported for several species, including *A. angustifolia* (Vieira et al. 2012) and *P. lambertii* embryogenic cultures (Fraga et al. 2016).

Here we describe the first SE protocol for a species from family Podocarpaceae, the *P. lambertii*. This protocol is based on induction with immature zygotic embryos, followed by establishment and multiplication of PEM on PGR-free culture medium, and maturation phases I and II. Procedures including explant preparation, PEM proliferation, and somatic embryo development are described in detail.

2 Protocol of Somatic Embryogenesis in *Podocarpus Lambertii*

2.1 Culture Medium

1. All culture medium used for *P. lambertii* SE is based on MSG basal salts (Becwar et al. 1988) and BM vitamins (Gupta and Pullman 1991). The detailed basal culture medium composition is described in Table 1.

Table 1 Stock solutions and culture medium composition for *Podocarpus lambertii* somatic embryogenesis

Constituents	Chemical formula	Stock Sol. ($g\ l^{-1}$)	Culture medium ($mg\ l^{-1}$)
Macrosalts 10X stock solution (use 100 ml per L of culture medium)			
Potassium chloride	KCl	7.45	745
Potassium nitrate	KNO_3	1.0	100
Calcium chloride dihydrate	$CaCl_2{\cdot}2H_2O$	4.4	440
Magnesium sulfate heptahydrate	$MgSO_4{\cdot}7H_2O$	3.2	320
Potassium phosphate monobasic	KH_2PO_4	1.7	170
Microsalts 100X stock solution (use 10 ml per L medium)			
Potassium iodide	KI	0.083	0.83
Boric acid	H_3BO_3	0.62	6.2
Manganese sulfate tetrahydrate	$MnSO_4{\cdot}4H_2O$	1.69	16.9
Zinc sulfate heptahydrate	$ZnSO_4{\cdot}7H_2O$	0.86	8.6
Sodium molybdate dihydrate	$Na_2MoO_4{\bullet}2H_2O$	0.025	0.25
Cupric sulfate pentahydrate	$CuSO_4{\cdot}5H_2O$	0.0025	0.025
Cobalt chloride hexahydrate	$CoCl_2{\cdot}6H_2O$	0.0025	0.025
Fe–EDTA 100X stock solution (use 10 ml per L medium)			
Iron sulfate heptahydrate	$FeSO_4{\cdot}7H_2O$	2.78	27.8
EDTA disodium salt	$Na_2EDTA{\cdot}2H_2O$	3.73	37.3
Vitamins 500X stock solution (use 2 ml per L medium)			
Nicotinic acid	$C_6H_5NO_2$	0.25	0.5
Pyridoxine hydrochloride	$C_8H_{11}NO_3{\bullet}HCl$	0.25	0.5
Thiamine hydrochloride	$C_{12}H_{17}ClN_4OS{\bullet}HCl$	0.50	1
Glycine	NH_2CH_2COOH	1.00	2
Other additives			
Myo-inositol	$C_6H_{12}O_6$		100
L-Glutamine	$H_2NCOCH_2CH_2CH(NH_2)CO_2H$		1460
Sucrose, maltose, Poly(ethylene glycol) average Mn 4000, glutathione, charcoal, abscisic acid, phytagel	According to the protocol step (Table 2)		
pH			5.8

2. According to the different protocol steps (induction, multiplication, maturation phase I and II) the culture medium should be supplemented with different compounds, which are detailed in Table 2.
3. Stock solutions must be prepared using distilled water and analytical grade reagents.
4. Macro-and microsalts stock solutions can be stored for several months in a refrigerator (4 °C) or in freezer (−20 °C) if storage has to last for longer periods. Aliquot vitamins stock solution and storage at −20 °C.

Table 2 Culture medium composition for *Podocarpus lambertii* somatic embryogenesis according to the different steps of the protocol

	Somatic embryogenesis step	Culture medium additives[1]
1.	Induction[2]	Sucrose (30 g l^{-1}), phytagel (2.5 g l^{-1})
2.	Establishment[2]	Sucrose (30 g l^{-1}), phytagel (2.5 g l^{-1})
3.	Multiplication (cell suspension)[3]	Sucrose (30 g l^{-1})
4.	Maturation phase I[4]	Maltose (50 g l^{-1}), Poly(ethylene glycol) avg Mn 4000 (100 g l^{-1}), glutathione (0.5 mM), activated charcoal (2 g l^{-1}), phytagel (3 g l^{-1})
5.	Maturation phase II[4]	Maltose (50 g l^{-1}), Poly(ethylene glycol) avg Mn 4000 (100 g l^{-1}), activated charcoal (2 g l^{-1}), abscisic acid (75 μM), phytagel (3 g l^{-1})

[1]Culture medium basal composition is fully described in Table 1
[2]In Petri dishes (90 × 15 mm) containing 25 ml of semi-solid culture medium, in absence of light at temperature of 22 °C
[3]Cell suspension is established with 500 mg of proembryogenic masses in 250 ml Erlenmeyer flasks containing 50 ml of liquid culture medium, in absence of light and permanent agitation (90 rpm) in orbital shaker at temperature of 22 °C
[4]In Petri dishes (90 × 15 mm) containing 25 ml of semi-solid culture medium, in low light intensity of 5–10 μmol m^{-2} s^{-1} at temperature of 22 ºC

5. Glutathione and ABA solutions should be prepared just before use and filter-sterilized.
6. Adjust the pH by adding drops of 0.5 M NaOH or 0.5 M HCl solution to reach pH 5.8. Autoclave for 15 min at 121 °C in an Erlenmeyer flask.

2.2 Somatic Embryogenesis Induction

1. The seed maturity stage in *P. lambertii* can be visually detected by the color of the fleshy receptacle adhered to the seed (Fig. 1). Immature seeds of *P. lambertii* are used for somatic embryogenesis induction, and its appearance can be visualized in Fig. 1a, b. The best induction results are obtained when globular-staged zygotic embryos are used (Figs. 1a, b and 2a, b).
2. For surface sterilization, remove the receptacle adhered to the seeds and wash them in running water. From here, all steps should be performed in laminar flow chamber. Immerse seeds in 70% ethanol solution for 3 min. Remove ethanol solution and add 2% sodium hypochlorite with 1 drop of Tween® 20 (Sigma-Aldrich, St Louis, MO) per 100 ml for 15 min. Remove sodium hypochlorite and wash seeds three times with autoclaved distilled water. All solutions must be added in enough volume to cover the seeds.

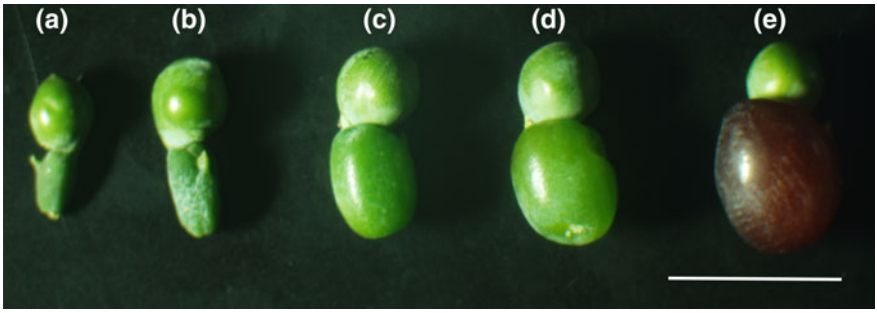

Fig. 1 *Podocarpus lambertii* seeds in different maturation stages. The fleshy receptacle adhered to the seed is brightly coloured at maturity (**e**). Seeds in maturation stages (**a**) and (**b**) are proper for somatic embryogenesis induction. Bar = 10 mm

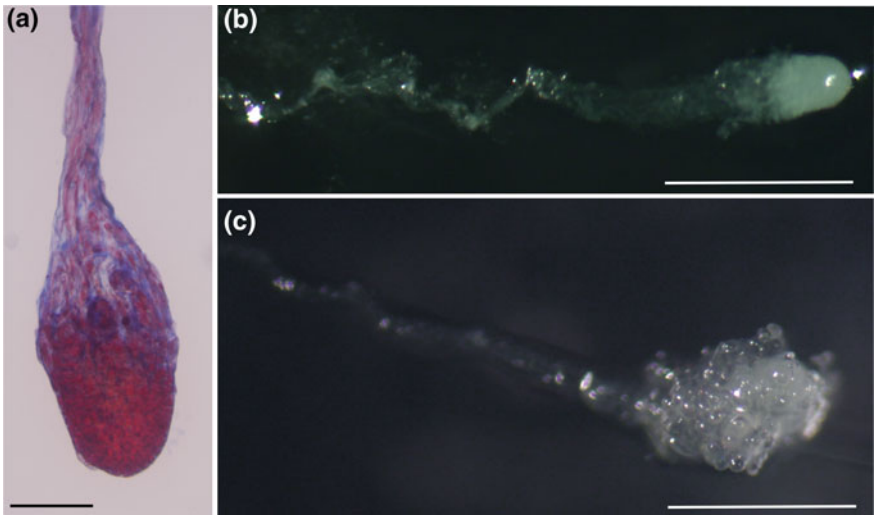

Fig. 2 *Podocarpus lambertii* somatic embryogenesis induction. **a** Globular zygotic embryo double stained with Evan's blue and acetocarmine. **b** Globular zygotic embryo inoculated in induction culture medium at day 0. **c** Proembryogenic masses formation at day 60. Bars: a = 250 μm; b and c = 1 mm

3. Excise the megagametophyte from the surface sterilized seeds with the aid of a scalpel and forceps under a stereomicroscope. Then, still with the aid of a stereomicroscope, excise the immature zygotic embryo and inoculate into Petri dishes (90 × 15 mm) containing 25 ml of induction culture medium (Tables 1 and 2), in absence of light at temperature of 22 °C. This procedure results in low contamination rates (<5%), so five embryos per Petri dishes can be used.

2.3 Proembryogenic Masses Establishment

1. After two months of somatic embryogenesis induction, PEM should be formed (Fig. 2c). Transfer this PEM to the establishment culture medium according to Tables 1 and 2.
2. Subculture the PEM every 15 days to a new establishment culture medium. After 3–4 subcultures the PEM is ready to be transferred to multiplication culture medium in cell suspension.

2.4 Cell Suspension Cultures

1. Inoculate 500 mg of PEM into 250 ml Erlenmeyer flask containing 50 ml liquid culture medium. For cell suspension establishment use multiplication culture medium (Tables 1 and 2).
2. Incubate cultures at 22 °C on an orbital shaker set to 90 rpm in the dark.
3. Subculture PEM every 15 days by totally replacing the culture medium. This procedure can be realized with the aid of a cell dissociation sieve (Sigma-Aldrich), 80 mesh screens. Capture the EC by pouring the culture medium with EC in proliferation in the cell dissociation sieve. Transfer 500 mg of EC to a new flask.

2.5 Maturation Phase I and II

1. The maturation phase I promotes the transition of PEMs to early somatic embryos. This transition is essential for an adequate response to ABA in maturation phase II.
2. After proliferation step in cell suspension, the PEM can be transferred to the maturation phase I culture medium. Using a 1–5 ml micropipete, collect 2 ml of cell suspension and spread above sterile filter paper disc (Ø 80 mm) in a Büchner funnel. Transfer the filter paper with the drained and dispersed EC to a Petri dish containing gelled maturation phase I culture medium.
3. The Petri dishes should be incubated in low light intensity 5–10 μmol m^{-2} s^{-1} at temperature of 22 °C for 30 days. At this step, PEM should look like Fig. 3a.
4. After 30 days, transfer the whole filter paper, containing the EC, to a new Petri dish containing maturation phase II culture medium. The Petri dishes should be incubated in low light intensity 5–10 μmol m^{-2} s^{-1} at temperature of 22 °C for 30 to 45 days.
5. After 30–45 days in maturation phase II culture medium, cotyledonary-and torpedo-staged somatic embryos should be formed (Fig. 3b).

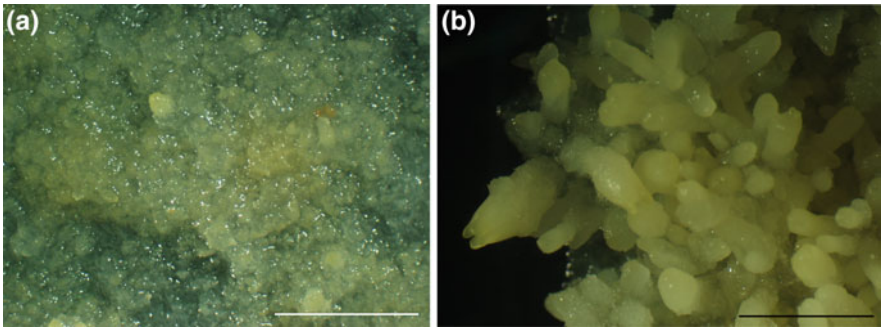

Fig. 3 Somatic embryogenesis maturation step. **a** Proembryogenic masses spread in the filter paper and inoculated in maturation phase I culture medium. **b** Somatic embryos after 30 days in maturation phase II culture medium. Bars: a = 5 mm; b = 2 mm

3 Cytochemical Analysis

1. Proembryogenic masses quality monitoring is essential in SE protocols. Only PEM with embryogenic features should be maintained and proliferated. The double staining protocol based on Evan's blue and acetocarmine is described below.
2. The double staining reveals the presence of the two typical embryonic conifer structures: the embryogenic cells, which are isodiametric and densely cytoplasmic, reacting in red with acetocarmine; and the suspensor-like cells, which are vacuolated and reacts in blue to Evan´s blue. The appearance of PEM during establishment and multiplication step can be visualized in Fig. 4a. Callus that does not show this features should be discarded.

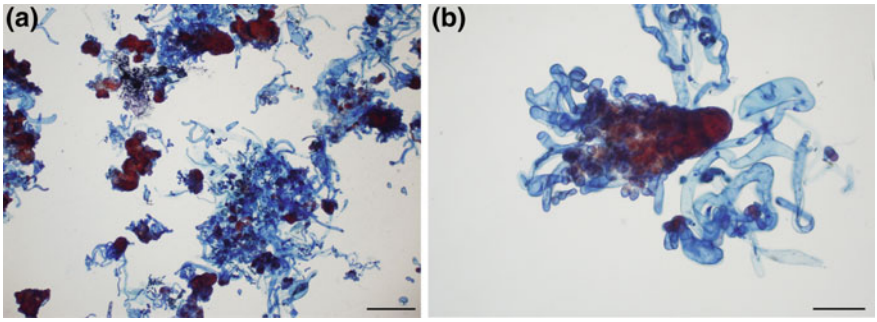

Fig. 4 Double staining with Evan's blue and acetocarmine. **a** Stained proembryogenic masses showing the presence of embryogenic cell (stained in red) surrounded by suspensor cells (stained in blue). **b** Early somatic embryo showing a polarized structure containing embryogenic and suspensor cells. Bars: a = 500 μm; b = 200 μm

3. The EC can also be monitored during maturation. The appearance of polarized early somatic embryo during maturation phase I can be visualized in Fig. 4b. The polarization of the early somatic embryos is essential for a good response to ABA in maturation phase II.
4. To perform the double staining with acetocarmine and Evan's Blue take an aliquot of PEM (~50 mg) and transfer to a watch glass. Add a drop of 1% acetocarmine (w/v) to the sample, gently mix and wait 1 min. Carefully remove the acetocarmine with the aid of toilet paper. Add a drop of 0.05% Evan's Blue (w/v) to the sample, gently mix and wait 1 min. Carefully remove the Evan´s Blue with the aid of toilet paper. Drop 1 ml of sterile distilled water. Visualize and examine an aliquot of the material under a light microscope.
5. The presence of embryogenic cells (stained in red) surrounded by some suspensor cells (stained in blue) should be visualized (Fig. 4a, b).

4 Research Prospects

P. lambertii is an important conifer species native from the Mixed Ombrophilous Forest ecosystem, which is part of the Brazilian Atlantic Forest biome. Its importance relies mainly in the maintenance of ecosystem stability as well as the valuable wood it provides. Somatic embryogenesis in this species is an appropriate biotechnological tool aiming at its conservation and propagation improvement. Here we describe for the first time an in vitro protocol based on somatic embryogenesis. The main steps of this protocol were successfully established, and the main bottleneck is the conversion step, which is currently under investigation. Ex situ conservation strategies, such as the establishment of a protocol for cryopreservation of embryogenic cultures, is also under investigation.

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Stress-Induced Microspore Embryogenesis by Anther Culture of *Quercus suber* L.

Pilar S. Testillano, Beatriz Pintos, Aranzazu Gomez-Garay and María C. Risueño

1 Introduction

In vivo, the microspore inside the anther divides and follows the gametophytic program to form the mature pollen grain. In vitro, upon the application of a stress treatment the microspore can be deviated towards a proliferation process leading to embryogenesis, the so-called microspore embryogenesis that can be induced in anther and isolated microspore cultures (Maluszynski et al. 2003). Currently, anther culture is the method of choice for microspore embryogenesis induction in many crops. Microspore-derived embryos can diploidize spontaneously (Testillano et al. 2004) or in response to chemical agents (Pintos et al. 2007), producing doubled-haploid embryos and plants (Bárány et al. 2005; Prem et al. 2012). Stress-induced microspore embryogenesis is a powerful biotechnological tool for plant breeding, as it is the fastest way to rapidly generate isogenic lines and new varieties via the production of doubled-haploid plants. This process is of much interest for the breeding of herbaceous and woody plants since it shortens the time and decreases the costs involved in breeding programs. In the case of woody species, tree breeding strategies have focused on ways to reduce the cycle time and improve the efficiency of selection; in this regard, genetic engineering approaches applied to haploid and doubled-haploid plants produced rapidly by in vitro microspore embryogenesis have a high potential. Nevertheless, due to their recalcitrance, the efficiency of embryogenesis in vitro systems in many woody species is still very low (Corredoira et al. 2017). Several studies have developed successful

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microspore embryogenesis in vitro systems in woody species (Germanà 2009; Germanà et al. 2006, 2011; Ramírez et al. 2003; Chiancone and Germanà 2016; Chiancone et al. 2015; Solís et al. 2008; Hofer 2004). However, efficient embryo germination and plant regeneration have been successfully achieved from microspore embryogenesis in very few woody species, e.g. Q*uercus suber* and *Citrus clementine*.

Quercus suber L. (cork oak) is a forest species of high economic and ecological value in the Mediterranean area. Cork oak supports a large industry that uses cork as the raw natural material for the production of wine bottle-stoppers or thermal and acoustic insulation products, as well as many other products. The induction of microspore embryogenesis in anther cultures from cork oak was first reported by Bueno et al. (1997). Since then, several studies have characterized different aspects of this in vitro system like the haploidy of embryos or the effects of some additives to the culture (Gomez et al. 2001; Gómez-Garay et al. 2009; Pintos et al. 2010; Bueno et al. 2000; Bueno and Manzanera 2003). Other reports have analysed the cellular changes associated with the microspore reprogramming to embryogenesis and have identified early embryogenic markers and key cellular processes, like MAPK expression, pectin esterification, auxin biosynthesis and DNA demethylation, involved in microspore embryogenesis initiation and progression in *Quercus suber* (Ramírez et al. 2004; Bueno et al. 2003; Rodriguez-Sanz et al. 2014).

Here we report an updated and detailed protocol to induce microspore embryogenesis in anther cultures of cork oak using a stress treatment of 33 °C. The protocol describes the selection of the appropriate (most responsive) microspore stage to initiate the culture; the pre-treatment of flower buds at 4 °C; the heat treatment of anthers; the in vitro conditions for induction, multiplication and maturation of embryos; and the conditions for embryo germination, plant conversion and acclimatization of regenerated plants.

2 Collection of Plant Material and Selection of Anthers

During the flowering period of *Quercus suber* (from early May to early-mid June), every week, branches with several catkins are cut and collected from trees in the countryside, near Madrid (El Pardo region). Cut tips of branches are immediately covered with moist cotton and aluminium foil, and transferred to the laboratory, where they are kept in the dark at 4 °C for several days, until use for in vitro culture.

To select the most appropriate anthers for embryogenesis induction, i.e. those containing microspores at the vacuolated stage [the most responsive stage for embryogenesis induction in most species, including cork oak (González-Melendi et al. 1995; Bárány et al. 2005; Ramírez et al. 2004; Prem et al. 2012)], anthers from catkins of different sizes are excised and processed for microscopic observation. Anthers are immersed in Carnoy fixative (ethanol:acetic acid, 3:1, v:v) for 2–18 h at 4 °C. They are then washed in water and immersed in a solution of 1 mg mL^{-1}

DAPI (4′,6-diamidino-2-phenylindole) for 15 min, squashed on slides and observed in an epifluorescence microscope under UV light. DAPI specifically binds to DNA, resulting in nuclei with blue fluorescence. A correlation between morphology-size of catkins and microspore developmental stage can be established and catkins containing microspores at the vacuolated stage are selected for in vitro culture. Although a certain developmental asynchrony occurs in microspores within anthers of the same flower, catkins that contain anthers with a major proportion of vacuolated microspores (Fig. 2b) are normally those that are around 15–20 mm long, slightly red in colour, with flowers of 2 mm diameter (Fig. 1a). The selected catkins contain green-yellow anthers that are 1.2–1.3 mm long (Fig. 1b).

Catkins in branches are pretreated at 4 °C for 4–7 days (maximum), the period in which microspores remain viable. During this period at 4 °C, anther culture can be set up on consecutive days. This procedure allows branches collected from the countryside on the same day to be used for one week for in vitro culture. It is thought that the pretreatment of branches contributes to the stopping of gametophytic development and the preparation of microspores for induction.

3 Anther Culture and Induction of Microspore Embryogenesis

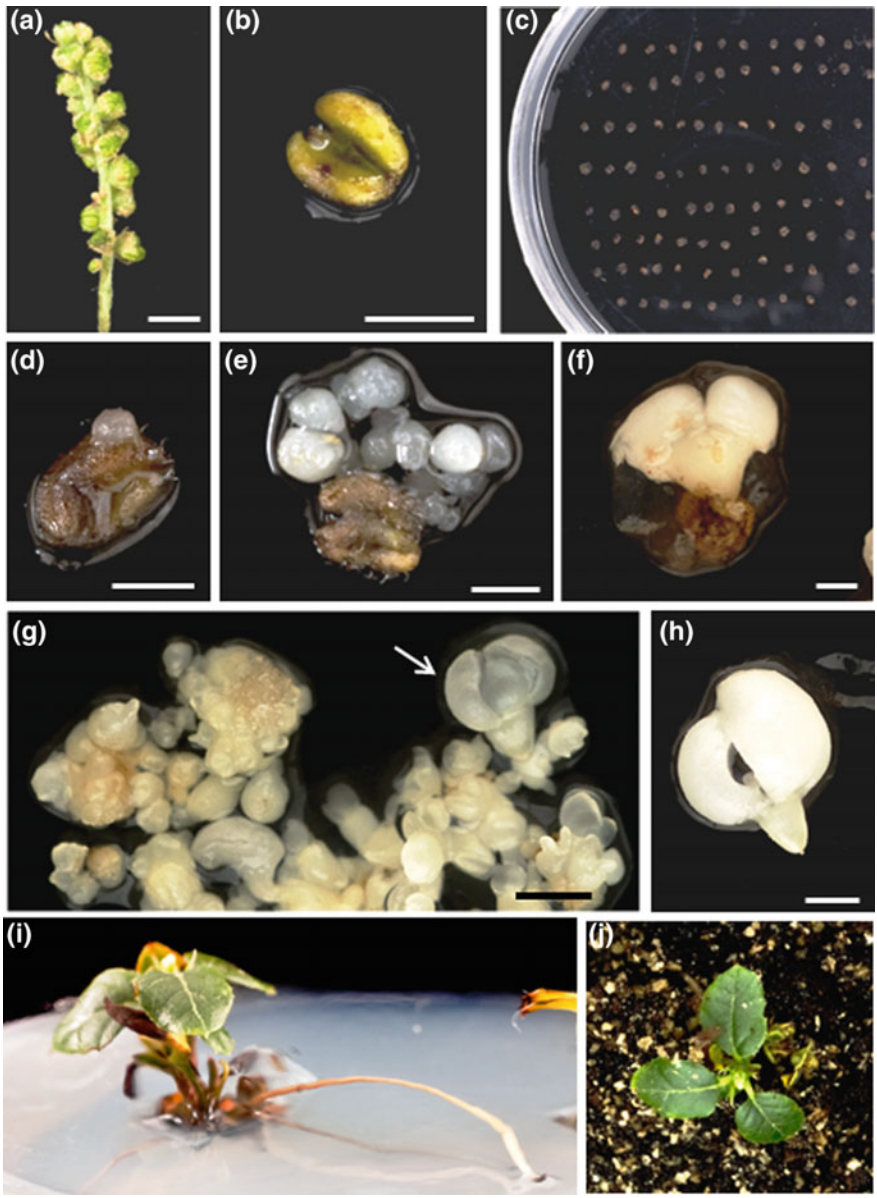
3.1 Sterilization

Selected catkins are separated from branches and sterilized by immersion in 70% ethanol for 30–60 s, under vacuum, to aid penetration of the solvent. They are then immersed in 2% sodium hypochlorite with 1% Tween-20 for 20 min, with magnetic stirring. After three washes in sterile distilled water, catkins are prepared for dissection and anther excision.

3.2 Anther Culture Initiation and Embryogenesis Induction

Anthers containing vacuolated microspores (Figs. 1b, 2a) are carefully excised from sterilized catkins under aseptic conditions and plated in Petri dishes of 90 mm diameter on solid **induction medium** (Table 1) which contains Sommer medium macronutrients (Sommer et al. 1975); Murashige and Skoog (MS) micronutrients and vitamins (Murashige and Skoog 1962); as well as 30 g/L sacarose and activated charcoal. No growth regulators are included in the induction medium. Anthers are placed in linear arrays of 10–12 anthers each, with a gap of around 5 mm between each anther, and up to 100 anthers per Petri dish (Fig. 1c).

Embryogenesis is induced by keeping the anthers at 33 °C in darkness for 5 days. After this inductive treatment, the anther cultures are transferred to 25 °C



and are still kept in darkness. Over the following 20–30 days, responsive anthers become swollen, and early proembryos are visible as very small white structures emerging from the anther interior (Fig. 1d), breaking the tissues of the anther wall which have become dark brown (Fig. 1d). After a few more days, proembryos grow and form globular embryos that can be found as white rounded structures over and

◂**Fig. 1** Main stages of stress-induced microspore embryogenesis in anther cultures of *Quercus suber* **a** Catkin with flower buds at the selected stage for induction. **b** Anther containing vacuolated microspores, the most responsive developmental stage, at the beginning of the culture. **c** Panoramic view of a plate showing the distribution of anthers in linear arrays, at the beginning of culture. **d** Responsive anther showing a proembryo emerging from the anther interior, around 20–30 days after induction. **e** Globular embryos (white rounded structures) over and around an anther (dark brown). **f** Immature cotyledonary embryo, formed by direct embryogenesis from an anther. **g** Embryos at different developmental stages and clumps of embryogenic masses, formed after several subcultures in proliferation medium; arrow points to an immature cotyledonary embryo. **h** Mature cotyledonary embryo, after culture in maturation medium. **i** Plantlet produced in vitro after germination of a mature embryo. **j** Plant regenerated and acclimatizated ex-vitro from microspore embryogenesis. Bars, A: 4 mm; B, D–F: 1 mm; G, H: 2 mm

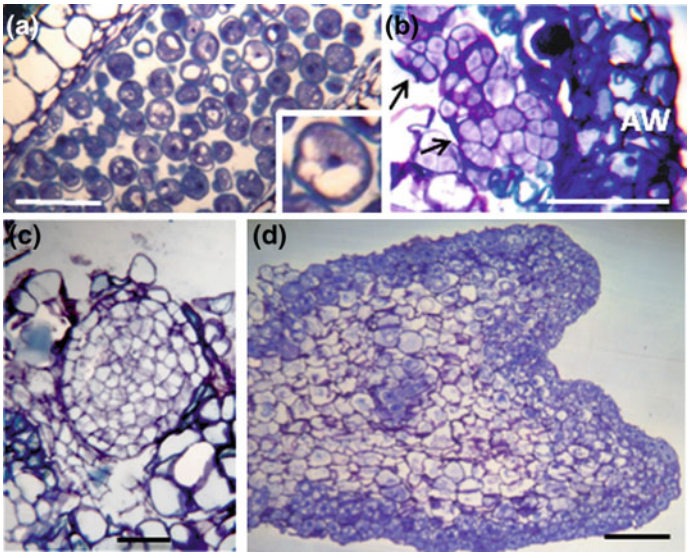


Fig. 2 Cellular organization along stress-induced microspore embryogenesis *Quercus suber*. Micrographs of semithin sections stained by toluidine blue and observed under bright field microscopy. Samples of microspore embryogenesis anther cultures at different stages, after fixation, Technovit resin embedding and semithin sectioning. **a** Anther containing vacuolated microspores, at the beginning of culture. Inset: High magnification of a vacuolated microspore. **b** Multicellular proembryos (arrows) formed inside the anther and surrounded by the tissue of the anther wall (AW). **c** Early globular embryo emerging from the anther tissues. **d** Developing heart-torpedo embryo. Bars, A–C: 50 µm; D: 100 µm

around the anthers (Fig. 1e). Several globular embryos can originate from each anther by direct embryogenesis from individual microspores (Fig. 1e). Over the days that follow, globular embryos develop to form heart-shaped, torpedo and cotyledonary embryos (Fig. 1f). Meanwhile, given that the response is not synchronized, new embryos can be formed from other anthers.

Table 1 Induction medium

Component	100× stock solution (g/L)	Final concentration (mg/L)
Sommer macronutrients		
KNO_3	100	1000
$NaH_2PO_4{\cdot}2H_2O$	12.95	129.5
$MgSO_4{\cdot}7H_2O$	25	250
$SO_4(NH_4)_2$	20	200
KCl	30	300
$CaCl_2{\cdot}2H_2O$	15	150
MS micronutrients		
$FeSO_4{\cdot}7H_2O$	2.78	27.8
Na_2EDTA	3.75	37.5
H_3BO_3	0.62	6.2
$MnSO_4{\cdot}H_2O$	1.69	16.9
$ZnSO_4{\cdot}7H_2O$	0.86	8.6
KI	0.083	0.83
$Na_2MoO_4{\cdot}2H_2O$	0.025	0.25
$CuSO_4{\cdot}5H_2O$	0.0025	0.025
$CoCl_2{\cdot}6H_2O$	0.0025	0.025
MS vitamins and aminoacids		
Glycine	0.2	2.0
Myoinositol	10	100
Nicotinic acid	0.5	5.0
Pyridoxine-HCl	0.5	5.0
Thiamine-HCl	0.1	1.0
Ascorbic acid	0.2	2.0
Other components		
Sucrose		30,000
Agar		8,000
Activated charcoal		10,000
pH 5.6		

At culture initiation, microscopic analysis of anther cultures reveals vacuolated microspores (Fig. 2a) that, after induction, divide producing small multicellular proembryos inside the anther (Fig. 2b). Later, early globular embryos can be found inside anthers (Fig. 2c), and emerging from anther wall tissues. After further growth, they separate from anther wall tissues, producing late globular, heart and torpedo (Fig. 2d) embryos which have a cellular organization and morphology that mimic zygotic embryogenesis (Bueno et al. 2003).

After one month, embryos develop to reach the cotyledonary stage. At this point, they are translucent, 2–3 mm long and have two large cotyledons (Fig. 1f). These immature cotyledonary embryos are individually transferred to new plates with medium for either embryo culture multiplication/proliferation or embryo maturation.

4 Multiplication of Microspore Embryo Cultures

Microspore-derived embryos, produced after induction and one month in culture, are transferred to new plates with **proliferation medium** (Table 2) which has a similar composition to the induction medium except that it does not contain activated charcoal and is supplemented with 0.5 g/L glutamine. They are kept at 25 °C in darkness and sub-cultured every month in the same medium, where embryos can spontaneously originate new embryos, by secondary recurrent embryogenesis, or produce embryogenic masses that later form new embryos (Fig. 1g). Using this procedure, microspore embryogenesis cultures can be clonally propagated to produce new embryos for several months. In proliferation medium, some of these embryos produced by indirect and secondary embryogenesis, can spontaneously differentiate until the stage of immature cotyledonary embryo, which have the same morphology as those produced by direct embryogenesis in anther cultures; they are translucent, 2–3 mm long and have two cotyledons (Fig. 1g, arrow).

5 Assessment of Ploidy Level of Embryos

To assess the ploidy level of embryos produced in anther cultures, a flow cytometry analysis of isolated nuclei is performed (Bueno et al. 2003). 10–50 mg of embryos are separated from anther culture plates and processed for all nuclei extraction. Globular to young cotyledonary embryos produced in proliferation medium are the most suitable stages for the analysis since, in more advanced embryo stages, large cotyledons contain abundant starch, which frequently makes the extraction procedure difficult.

For flow cytometry analysis, embryos can be either previously fixed in 4% paraformaldehyde or directly homogenized without fixation. Embryo samples (10–50 mg) are treated with 2 mL lysis buffer (5 mM Tris, 2 mM Na_2EDTA, 80 mM KCl, 20 mM NaCl, 0.1% Triton X-100 in deionized water, pH 7.5) and homogenized over an ice bath by manual chopping with a razor blade. After homogenization, the solution is filtered through a 30 µm nylon mesh, and the resulting filtrate is transferred into 2 mL Eppendorf tubes and centrifuged (600*g*, 4 °C). The pellet is resuspended in 500 µL of lysis buffer and the nuclei suspension is transferred to cytometer tubes and treated with 55 µL of propidium iodide solution (1 mg/mL in PBS containing 15 mg/mL of RNase). Nuclei (n= 10,000) from each sample are measured in an Epics-xl flow cytometer (Coulter). As a control, a nuclei suspension from diploid parental plants is used.

Flow cytometry analysis reveals that most embryos produced in cork oak anther cultures are haploids (between 80 and 91%), a few are diploid (between 5.7 and 12%) and fewer still have a mixture of haploid and diploid nuclei (Bueno et al. 2003; Pintos et al. 2007). These results indicate that there is a very low proportion of spontaneous diploidization in microspore embryos in this species, and it is

Table 2 Proliferation medium

Component	100× stock solution (g/L)	Final concentration (mg/L)
Sommer macronutrients		
KNO_3	100	1000
$NaH_2PO_4{\cdot}2H_2O$	12.95	129.5
$MgSO_4{\cdot}7H_2O$	25	250
$SO_4(NH_4)_2$	20	200
KCl	30	300
$CaCl_2{\cdot}2H_2O$	15	150
MS micronutrients		
$FeSO_4{\cdot}7H_2O$	2.78	27.8
Na_2EDTA	3.75	37.5
H_3BO_3	0.62	6.2
$MnSO_4{\cdot}H_2O$	1.69	16.9
$ZnSO_4{\cdot}7H_2O$	0.86	8.6
KI	0.083	0.83
$Na_2MoO_4{\cdot}2H_2O$	0.025	0.25
$CuSO_4{\cdot}5H_2O$	0.0025	0.025
$CoCl_2{\cdot}6H_2O$	0.0025	0.025
MS vitamins and aminoacids		
Glycine	0.2	2.0
Myoinositol	10	100
Nicotinic acid	0.5	5.0
Pyridoxine-HCl	0.5	5.0
Thiamine-HCl	0.1	1.0
Ascorbic acid	0.2	2.0
Other components		
Glutamine		500
Sucrose		30,000
Agar		8,000
pH 5.6		

necessary to apply additional treatments with diploidization agents, e.g. anti-mitotics (Pintos et al. 2007), to increase the production of doubled-haploid embryos in this system.

6 Maturation of Microspore Embryos

Both types of immature cotyledonary embryos—those produced by direct embryogenesis (Fig. 1f) and those formed by indirect or secondary embryogenesis, after culture in proliferation medium (Fig. 1g)—can be separated and cultured on

Table 3 Maturation medium

Component	100× stock solution (g/L)	Final concentration (mg/L)
Sommer macronutrients		
KNO_3	100	1000
$NaH_2PO_4{\cdot}2H_2O$	12.95	129.5
$MgSO_4{\cdot}7H_2O$	25	250
$SO_4(NH_4)_2$	20	200
KCl	30	300
$CaCl_2{\cdot}2H_2O$	15	150
MS micronutrients		
$FeSO_4{\cdot}7H_2O$	2.78	27.8
Na_2EDTA	3.75	37.5
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$MnSO_4{\cdot}H_2O$	1.69	16.9
$ZnSO_4{\cdot}7H_2O$	0.86	8.6
KI	0.083	0.83
$Na_2MoO_4{\cdot}2H_2O$	0.025	0.25
$CuSO_4{\cdot}5H_2O$	0.0025	0.025
$CoCl_2{\cdot}6H_2O$	0.0025	0.025
MS vitamins and aminoacids		
Glycine	0.2	2.0
Myoinositol	10	100
Nicotinic acid	0.5	5.0
Pyridoxine-HCl	0.5	5.0
Thiamine-HCl	0.1	1.0
Ascorbic acid	0.2	2.0
Other components		
Sucrose		30,000
Agar		8,000
Activated charcoal		10,000
pH 5.6		

maturation medium (Table 3). Embryo maturation medium has a basal composition, without glutamine, with Sommer macronutrients and MS micronutrients and vitamins, plus 1% activated charcoal. Embryos progressively mature in darkness for 4 weeks at 25 °C. After this period, mature embryos have accumulated reserve nutrients and cotyledons have markedly increased in size, and have a white opaque appearance, with well-formed embryogenic axis and hypocotyl (Fig. 1h).

7 Microspore Embryo Germination and Plant Conversion

To improve germination, mature embryos are vernalized at 4 °C for 2 months, in maturation medium. Embryos are then transferred to Petri dishes with **germination medium** (Table 4) and kept at 25 °C, with a photoperiod of 16/8 h for several weeks. Germination medium contains 6-benzylaminopurine (BA) and indole-3-butyric acid (IBA) as growth regulators; 15 g/L sucrose (lower concentration than maturation medium); and 10 g/L agar.

Table 4 Germination medium

Component	100× stock solution (g/L)	Final concentration (mg/L)
Sommer macronutrients		
KNO_3	100	1000
$NaH_2PO_4 \cdot 2H_2O$	12.95	129.5
$MgSO_4 \cdot 7H_2O$	25	250
$SO_4(NH_4)_2$	20	200
KCl	30	300
$CaCl_2 \cdot 2H_2O$	15	150
MS micronutrients		
$FeSO_4 \cdot 7H_2O$	2.78	27.8
Na_2EDTA	3.75	37.5
H_3BO_3	0.62	6.2
$MnSO_4 \cdot H_2O$	1.69	16.9
$ZnSO_4 \cdot 7H_2O$	0.86	8.6
KI	0.083	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.025	0.25
$CuSO_4 \cdot 5H_2O$	0.0025	0.025
$CoCl_2 \cdot 6H_2O$	0.0025	0.025
MS vitamins and aminoacids		
Glycine	0.2	2.0
Myoinositol	10	100
Nicotinic acid	0.5	5.0
Pyridoxine-HCl	0.5	5.0
Thiamine-HCl	0.1	1.0
Ascorbic acid	0.2	2.0
Growth regulators		
BA		0.05
IBA		0.1
Other components		
Sucrose		15,000
Agar		10,000
pH 5.6		

Under these conditions, embryos germinate and develop roots, and cotyledons turn green. When they exhibit a well-formed radicle, embryos are transferred individually to a glass culture vessel to provide more space for further growth. The vessel contains the same germinating medium. Over the following days, under the same culture conditions, embryos convert into plantlets with true leaves and large roots (Fig. 1i). After 6–8 weeks of growth inside the vessels in vitro, the plantlets develop new leaves and secondary roots; it is at this point that they can be acclimatized.

8 Plant Acclimatization

In vitro plantlets that have a well developed root system are carefully extracted from vessels and cultured ex-vitro for hardening and acclimatization. They are washed to remove the agar and transferred to trays with a sterile potting mix of peat:vermiculite, 3:1. Trays are covered with a transparent plastic film to maintain a high humidity environment. Plantlets in trays (Fig. 1j) are transferred to a growth chamber with controlled temperature of 25 °C and a photoperiod of 16 h light. After several weeks of progressively decreasing relative humidity, plants are transferred to pots with the same peat:vermiculite mix and placed in the greenhouse, where they further harden and acclimatize.

9 Concluding Remarks

In this chapter, we report an updated protocol of stress-induced microspore embryogenesis in anther culture of cork oak. The method reported describes the selection of the appropriate (most responsive) microspore stage to initiate the culture; the in vitro conditions for induction, multiplication and maturation of embryos; as well as the conditions suitable for embryo germination, plant conversion and acclimatization of regenerated plants. Critical conditions for efficient embryogenesis induction are as follows: vacuolated microspores as initial stage for anther culture, pre-treatment of flower buds at 4 °C for a few days, and stress treatment of anthers at 33 °C for 3 days in culture medium that is free of growth regulators. The in vitro system reported here is one of the very few available methods in a woody species in which induction of microspore reprogramming; embryo development and maturation; embryo germination; and plant regeneration have all been successfully achieved from anther culture. Despite the high potential of doubled-haploids in tree breeding strategies, there are still many tree species that have very low rates of microspore embryogenesis efficiency or that are even completely recalcitrant to the process. Further work is required to identify the cellular and molecular mechanisms underlying microspore embryogenesis induction and progression, especially in woody species in which information is scarce.

In this regard, cork oak microspore embryogenesis could be used as a model system to investigate the process in trees, as reported in our previous publications. The understanding of the cellular processes that operate during induction and progression of microspore embryogenesis will help to manipulate the process more efficiently to achieve higher embryo productivity in forest species of interest.

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Manufactured Seeds of Woody Plants

Jeffrey E. Hartle

Though I do not believe that a plant will spring up where no seed has been, I have great faith in a seed. Convince me that you have a seed there, and I am prepared for wonders.
Henry David Thoreau

1 Introduction

Redenbaugh (1986, 1993) defined a synthetic seed as a somatic embryo inside a coating, and as being directly analogous to a zygotic seed. There have been several names given such "seed" including artificial seed, synthetic seed, seed analog and somatic embryo seed. We believe that the term "manufactured" seed, reflects the nature of the construct in a more accurate way. The practical requirements of such seed are that they perform the basic functions of a botanic seed during the sowing and germination of a somatic embryo under field conditions. These basic functions include: protecting the embryo and surrounding nutritive matrix from mechanical damage, desiccation and microbial invasion; providing for an adequate supply of nutrients including carbon, gas exchange, and water to support germination; and physical properties that allow the germinating embryo to emerge normally from the seed under field conditions.

Many woody plants can be vegetatively propagated to allow genetic gain from maintaining traits that would be lost during meiotic recombination. Another substantial benefit in the production of vegetatively propagated woody plant crops is the opportunity to harvest uniform material. Increased raw material uniformity will open many new opportunities to make wood-based-manufacturing facilities more efficient.

With genetic engineering becoming increasingly less expensive and potentially a functional part of genetic improvement programs, the role of manufactured seed

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could be critical to success in at least two ways. First is the delivery of transgenic foundation stock. Secondly, if regulations mandate the use of sterile plants for field culture of transgenic crops then manufactured seed offer an economical method of delivery into the agronomic system.

Once a decision is made to vegetatively propagate a given woody plant, then the next decision is which method to use. Rooted cuttings can be used with many plants. There are commonly severe limitations to the number of propagules that can be produced per genotype. This is due to reduction in percentage of cuttings that root, and in some cases also reduced growth rate as the stock plant age increases. Also with many rooted cutting systems costs are limiting. Organogenesis offers another method of clonal multiplication, but costs are prohibitive because of the labor intensive nature of the process. Theoretically, somatic embryogenesis offers the potential for a low cost vegetative propagation method that allows a very large number of embryos to be propagated per genotype (not biologically limited) and allows cryopreservation of cultures while genotypes are being evaluated. Krikorian (1988), Bornman (1991), Mo (1993) and Redenbaugh (1993) pointed out that the quality of somatic embryos does not allow practical realization of this potential efficiency. Goebel-Tourand et al. (1993) demonstrated some of the consequences of poor somatic embryo morphology on normalcy during conversion. Though the references are older, the issues of somatic embryo quality still exist. Commercially available conifer seedlings produced via somatic embryogenesis are almost five times more costly than open pollinated sources and one and a half times more costly than the best control pollinated sources (Arborgen LLC sales catalog). This cost differential is indicative of the quality and number of embryos the process can create. Once somatic embryos begin to reach the quality necessary to produce zygotic-embryo-like vigor, then it will be possible to move forward toward more cost effective commercialization. Manufactured seed offer the potential of delivering somatic embryos to the field or greenhouse utilizing standard agronomic, horticultural and forestry practices for seed sowing and crop culture. The technology will be very valuable at implementation but economical implementation demands major improvements in somatic embryo quality and in manufactured seed performance under normal agronomic conditions.

2 Historical Development of Manufactured Seed

Woody plant utilization of the manufactured seed concept arises out of earlier concepts applied to agronomic plants. There have been two schools of thought, desiccated and hydrated. We will, therefore, briefly summarize the historical derivation for the reader's reference. For a more complete discussion we refer the reader to Redenbaugh (1993).

Desiccated manufactured seed were first developed by Kitto and Janick (1986) at Purdue University. This seed consisted of desiccating an embryo coated with Polyox®. Germination, albeit at low levels, was achieved by placing the dried wafer

in tissue culture media then plating on media moistened filter paper for germination. This approach was followed by Gray at the University of Florida, and Attree and Fowke at the University of Saskatchewan at Saskatoon. Current reports on the use of this technology are rare in the modern literature.

Hydrated manufactured seed were first developed by Redenbaugh (1986). This method involves encapsulation of the embryo in a drop of sodium alginate followed by complexing with calcium ions to form a calcium alginate gel. Most of the published research on manufactured seed involves either this method used directly or with modifications. Communication of research and development of this alginate bead seed analog technology by Redenbaugh and associates galvanized the imagination of potential user groups and stimulated much research on a world-wide level. Most current publications still report the use of alginate bead seed as an artificial seed.

3 Practical Considerations in the Design of Manufactured Seed

Natural seed have many functional features that facilitate their use in agriculture: (1) a hard seed coat that provides protection from mechanical damage, reduces the rate of drying and slows microbial invasion, (2) an endosperm, or in conifers a female gametophyte, that provides a controlled release of nutrients and an energy source, and physically constrains the germinating embryo such that elongation of the embryo promotes emergence of the radicle, then hypocotyl and finally the cotyledons from the seed, (3) an anatomically, physiologically and biochemically correct embryo which germinates and grows with substantial vigor, and (4), the capability to withstand wide variation of temperature, soil moisture, oxygen availability, and microbial inoculum levels to yield high germination. Manufactured seed must mimic natural seed functions if they are to perform in the agricultural field in a manner that will allow economical application of the technology (Fig. 1b). Just as low vigor zygotic seed is often observed to undergo mortality from several secondary causal factors such as microbial invasion, manufactured seed must be vigorous to avoid a similar fate.

4 Current Status of Design of Manufactured Seed

4.1 Manufactured Endosperm or Female Gametophyte

Natural seed show a large variation within and among genera in the proportion of the total seed nutrient and energy reserves stored in the embryo. Some species require extra-embryo reserves from the endosperm/female gametophyte to

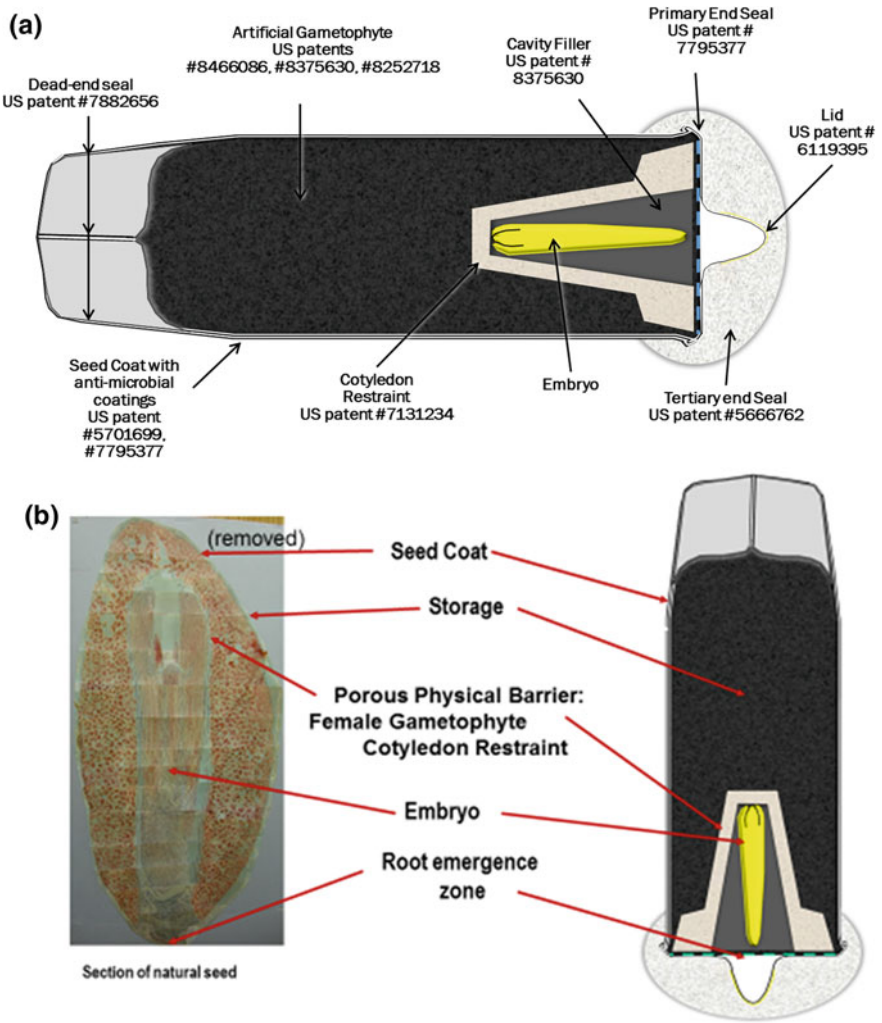

Fig. 1 **a** Weyerhaeuser Company design for manufactured seed, **b** comparison of Weyerhaeuser Co. manufactured seed versus natural conifer seed

germinate while others will germinate under ideal conditions without it. A simple test of germinability of excised embryos on sterile water agar will give an initial indication of whether the species in question will germinate without female gametophyte/endosperm, that is whether an artificial gametophyte (or artificial endosperm) is necessary. Fujii et al. (1992) demonstrated, for example, that naked alfalfa embryos placed in the field under styrofoam cups had a 25% conversion to autotrophic plants. Nature is extremely efficient, providing for controlled enzymatic cleavage of complex molecules to supply simpler molecules to the surfaces

surrounding the embryo. In manufactured seed there are three choices in design of a system to supply nutrients: (1) limit nutrients to those carried in the embryo (i.e. no additional provided in the manufactured seed), (2) provide controlled release chemistry in the nutritive gel surrounding the embryo, or (3) provide a static supply of nutrients in the surrounding gel matrix but protect them from the leaching effects of water moving through the soil.

Redenbaugh et al. (1986) manufactured seed as used on alfalfa and celery embryos usually utilize the first approach. No additional nutrients are supplied outside the somatic embryo. Embryo development protocols that induce the development of high levels of reserves in the somatic embryo increase manufactured seed germination (Fujii et al. 1992). This approach is simple but it offers limited application, since most species either will not germinate without additional nutrients or have reduced vigor.

Kirin Brewing Company pioneered the use of controlled release nutrients. Sanada et al. (1993) reported increased conversion frequencies of carrot and celery embryos when microencapsulated sucrose was included in the alginate manufactured seed. Microcapsules were 0.5 mm in diameter and were coated with ethylene-vinyl acetate copolymer and wax. They released sucrose over several weeks and stopped releasing at low temperatures. The microencapsulation technology was reported in Sakamoto et al. (1991a). This technology was patented in 1989 (Sakamoto et al. 1991b). In the patent examples, germination of celery manufactured seed was improved from 30% without microcapsules to 90% with microcapsules when seed were germinated in vitro on a sugar free nutrient medium. Friend (1993) reported no improvement in conversion of alfalfa manufactured seed using sucrose microencapsulated with either cellulose acetate butyrate or gelatin. He suggested that low encapsulation efficiency could have caused the poor results apparently due to rapid release of sugar.

The third approach, involving freely available nutrients, has been used by many researchers beginning with Redenbaugh (1986) but the conditions of use define the result. Most of these tests utilize the calcium alginate 4 mm diameter bead seed design. If the embryo was dependent on the surrounding gel for nutrients, then results were generally good only if the experiments were done with the seed sown on the surface of nutrient media, or in soil irrigated with such media. The results were generally poor if the seed were sown in a system where no additional nutrients were available. This was due to the fact that the volume of the 4 mm diameter alginate bead was inadequate to hold the required nutrient supply or the nutrients were leached from the bead into the environment. This points out that it is critical to conceptualize use under agronomic conditions when determining the approach one will utilize. Germination on nutrient media can aid in defining some aspects of manufactured seed design, but the inference space of such experiments is very limited. For example, the effect of adding or subtracting a given nutrient can be studied in this way but the volume of the nutrient necessary to support germination in soil cannot.

Bapat and Rao (1988), and Bapat (1993) reported inclusion of nutrient salts in manufactured sandalwood seed which were beads of a composite of alginate and silica gel. Theoretically, silica gel should adsorb nutrients, acting to control their release to the embryo. In most experiments germination tests were carried out on nutrient media solidified with agar, thus nutrient depletion was not an issue. Where tap water was used, instead of nutrient media, germination was severely reduced. Similarly, Lulsdorf et al. (1993) added activated charcoal to alginate beads encapsulating interior spruce (*Picea glauca engelmannii* complex) and black spruce (*Picea mariana* Mill.). Such seed survived one month cold storage at 4 °C on nutrient media, although results varied between species and between zygotic and somatic embryos. All germination was carried out on solid nutrient media plates, thus again nutrient supply was not an issue.

Mukunthakumar and Mathur (1992) obtained germination of male bamboo with encapsulation in 6% calcium alginate with MS salts with or without 3% sucrose. They coated seeds with paraffin oil to reduce microbial invasion and desiccation. Germination was 45% in initially sterile soil in covered pots under greenhouse conditions in spite of heavy microbial invasion of the alginate. The investigators attribute this success to the anatomy and disease-resistant properties of bamboo.

At Weyerhaeuser, Carlson and Hartle (unpublished data) anticipated the need to supply conifer embryos with nutrients over the entire 6 week worst case potential germination period. They measured the nutrient use of conifer embryos and cross checked the estimate from the dry weight gain over the germination period. They calculated that the weight of nutrients necessary to support a "worst case" germination period would be approximately 6 mg. total salts including sugar. They tested several nutrient concentrations and determined that a gel volume of approximately 0.5 ml. would support conifer embryos over the 6 week period. This volume provides both water and nutrients to support the germination process.

4.2 Oxygen Supply in Hydrated Gels

In many manufactured seed designs oxygen has been noted as limiting to germination. As gel volumes become larger this problem becomes more acute. Carlson et al. (1993) dramatically improved the performance of manufactured seed by making oxygen more available through the inclusion of oxygen carrier emulsions in the gel matrix. This increased the number of normal germinants four fold in alginate bead seeds germinated on agar media which included nutrient salts. Most importantly, it also makes possible the use of large manufactured gametophyte/endosperm gel volumes to support the embryo through a potentially long germination period in soil (Fig. 1).

4.3 Manufactured Seed Coats

Hydrated gels used for manufacturing analogs of female gametophyte/endosperm rapidly loose water to the ambient air or soil on sowing. Practical application of manufactured seed requires that this water loss be controlled. Similarly, mechanical damage from sowing equipment and entry of soil microbes must be reduced by a coating, if high vigor is to be expected under practical farming conditions.

Friend (1993) reviewed work done at SRI International in conjunction with Redenbaugh's group at Plant Genetics Inc. to develop hydrophobic coatings for alginate bead manufactured seed. Specifically, ethylene vinyl acetate copolymers of various types can be applied to the gel surface by spraying, dipping or immersion in a solution of the polymer in a solvent. Since the organic solvents suitable to the process are highly phytotoxic, it is very important to control the process carefully such that solvents do not penetrate to the depth of the embryo. If done carefully coatings can be applied without reducing the shoot emergence of alfalfa embryos from the alginate beads (Friend 1993). Moisture loss can be controlled to less than 1% per day by such methods.

Natural seed have relatively hard seed coats that prevent rapid water loss and as well protect the seed from mechanical damage during handling, mechanical sowing and in the soil. Carlson et al. (1993, 1995) and Hartle et al. (2007) have worked to develop a hard seedcoat that provides adequate gas exchange, but is water impermeable. The synthetic seed coat these authors developed also prevents mechanical damage to the embryo lying within. Initially the authors developed a wax impregnated paper seed coat, but eventually abandoned that structure for a more simply manufactured biodegradable plastic structure (Fig. 2). This coat has many of the favorable properties of a natural seed coat, is harder than the coating mentioned above, and is easily applied without exposing the manufactured gametophyte/endosperm or embryo to risk of organic solvent induced damage.

Fig. 2 Weyerhaeuser Company manufactured Douglas-fir seed (Carlson et al. 1993) in various stages of germination. In this case manufactured seeds contained excised zygotic embryos. From L to R; newly emerging germinant in the "crook stage" of development, seeds were sown 6 mm under the soil surface; Emergent germinant raising the seed coat above the soil. As with natural seed, manufactured seed sometimes emerge leaving the coat under the soil surface, and sometimes shed the coat as shown here; Germinant has shed the seed coat and is free to grow

4.4 Physical Considerations in Germinant Emergence from Manufactured Seed

Natural seed anatomy provides a direct exit for the root cap as the embryo begins to elongate during germination. As root, hypocotyl and cotyledon elongation occur, the root cap, then root apex, then hypocotyl and finally cotyledons emerge along an anatomically defined route. Physical forces of elongation result in progressively shedding the seed coat including any remaining nutritive tissues (Fig. 2).

Hydrogel encapsulation can produce gels that are soft enough to allow penetration by the root cap and germinant emergence. Dehydration of the hydrogel aids in emergence. If the seed coat is hard enough to provide mechanical protection during mechanized sowing there is a potential for reduced embryo emergence due to the radicle curving along the inside of the seed coat rather than emerging through it. Similarly if the volume of gel is large enough to support the embryo through a several week germination period then there can be problems with the enlarging cotyledons becoming trapped in the gel rather than shedding the seed coat.

Redenbaugh et al. (1993) reviewed a method for making self-breaking capsules as a modification of the alginate bead technology. Such capsules break when they are exposed to water after sowing. Sakamoto (1994) was issued a patent for a self-breaking capsule. It was described as an interior complexed alginic acid gel core. The core was coated with a hard shell described as a polyamino acid, basic polysaccharide, bis biguanide, basic polymer containing an ion exchanger comprised of cellulose, and ion exchange resin, inorganic ion exchanger and polymer coagulate. This design too was described as breaking spontaneously on sowing in the soil.

Self-breaking capsule designs would indeed allow the embryo to emerge easily but have the disadvantage that many of the benefits of manufactured seed are lost at the point of breakage, leaving the embryo to germinate in loose contact with the gel which would then readily desiccate.

To prevent the germinant from penetrating and becoming trapped in the gel, Carlson et al. (1997) enclosed the embryo in a cotyledon restraint. This restraint orients the forces of the elongating embryo such that rapid germinant emergence is promoted (Fig. 2). Elongation of the radicle forces the root cap through a dimpled thin film that is sealed over the end of the cotyledon restraint. This design is analogous to natural seed in that the germinant does not shed the seed coat until nutrient reserves and protection from desiccation are no longer necessary because it has become autotrophic. This restraint can be customized for individual plant species and designed to match the size and shape of the somatic embryo the manufactured seed is designed for.

In natural seed the gametophyte and embryo develop together. This creates a close relationship between the gametophyte and embryo that facilitated nutrient transfer between these entities. With manufactured seed, the embryo is developed separately from the seed construct. The restraint described above is designed as a one size fits all part. This is required for high speed, low cost manufacturing. To

create an environment similar to natural seed where nutrient transfer is not limiting, a cavity filler can be used. In a US patent, Hartle et al. (2014c) developed a fill material that consists of a nutrient loaded adsorbent. This filler is used to fill the space not occupied by the embryo. It facilitates nutrient and water transfer from the synthetic gametophyte to the embryo. With this filler, a range of embryo sizes can be placed in a manufactured seed without customization.

So as not to inhibit germination, the dimpled thin film developed to cover the cotyledon restraint designed by Carlson et al. (1997) is easily penetrated by the elongation of the embryo as it germinates. This fact also makes the film susceptible to rupture via mechanical handling. To prevent premature rupturing of the film, Carlson et al. (2006) developed a coating that is applied over the dimpled film. This coating applied as a highly viscous water based solution that once dried creates a hard barrier that protects the thin film and allows manufactured seed to be handled like traditional seed. When manufactured seed are sown and irrigation has begun, the coating softens and becomes easily penetrable by the elongating embryo thus not inhibiting the germination process. This coating can also contain adjuvants beneficial to promoting germination such as pesticides or fertilizers.

5 Woody Plant Manufactured Seed

Is the design of manufactured seed for a woody plant species really different from that of a crop plant? We believe that there is little difference in the requirements that can be noted. Greenhouse testing of manufactured seeds performed with excised zygotic embryos of various species has shown the potential of manufactured seed to be used with somatic embryos of corn, wheat and soy (Fig. 3). With modifications to the cotyledon restraint design to match the embryo morphology of these species, Hartle et al. (2013a, b, c, d, e, 2014a, c) and Cootsona et al. (2013) have shown the potential of manufactured seed as designed for use with conifer somatic embryos to support good germination for other species. To optimize performance, it is likely that there will be some minor adjustment in contents and volume of the artificial female gametophyte or endosperm as different species are considered, but our preliminary testing shows that there are no major differences in seed design required for different species.

6 Benefits of Manufactured Seed Over Traditional In Vitro Germination Techniques

Conversion of in vitro germinated plants often requires an acclimation step to allow the germinants to survive the transition to greenhouse or field conditions. Plants are removed from the in vitro germination environment and planted in small, low volume pots. The plants are then usually grown for a time under greenhouse

Fig. 3 Weyerhaueser Co. manufactured seed germination of embryos from various species. Germination was under greenhouse conditions. Clockwise from top left: zygotic wheat; somatic Douglas-fir; zygotic soybean; zygotic sweet corn; somatic loblolly pine

conditions where temperature, light and humidity can be controlled. This phase is usually characterized by slow or lack of growth while the plant adjusts to the environment. Certain species can experience high mortality at this stage. Once growth has resumed, plants can be transplanted to a final larger container or bare root nursery bed. Again, there is a potential lag phase where growth is slowed as the plant adjusts. With the use of manufactured seed these acclimation steps can be avoided or reduced in timespan. Plants appear to either acclimate faster or do not require this process. In paired comparisons where somatic embryos of Douglas-fir (*Pseudotsuga menziesii*) were germinated either from manufactured seed in a greenhouse or via the in vitro germination and acclimation process described above, the plants at the same point in time were larger when germinated from manufactured seed than when germinated in vitro (Fig. 4).

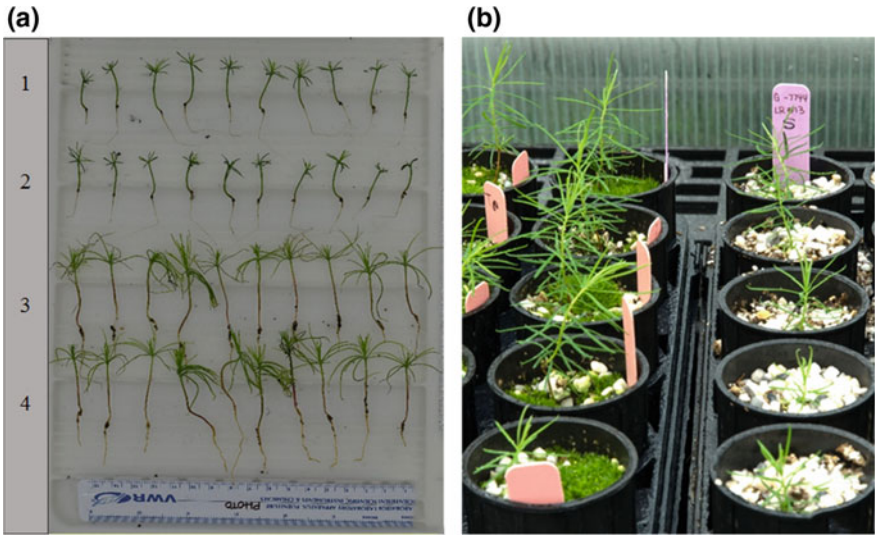

Fig. 4 Paired comparisons of germinants produced via Weyerhaeuser Co. manufactured seed versus traditional processes (in vitro germ followed by transplanting to non-sterile miniplug, then finally to pasteurized potting mix. **a** Zygotic embryos germinated in vitro (rows 1 & 2) or from manufactured seed sown in sterile sand (rows 3 & 4). **b** Douglas-fir somatic embryos germinated from manufactured seed in non-sterile potting mix (left) or via traditional process (right). In vitro germination media based off Murashige and Skoog (1962)

7 Current Limitations to Implementation of Manufactured Seed

7.1 Embryo Quality Must Improve

Somatic embryo quality must improve to the point where embryos have the same characteristic vigor and morphology as zygotic embryos. There has been continuous improvement in embryo quality in many species, but more work is needed to achieve zygotic-like quality.

7.2 Experimentation Must Be Carried Out Under Appropriate Conditions

Much current manufactured seed literature describes experiments in which embryos are encapsulated in alginate and germinated on nutrient media in vitro. These experiments do little to advance the state of the art. Experiments designed to evaluate the presence or concentration of components of an artificial female gametophyte/endosperm can be done on the surface of media, but one must realize

that no knowledge of the volume required to support the embryo and related factors will be gained. Similarly when testing the whole seed structure it should be sown at a normal depth in soil.

We have previously stated that there is some variation among species in response of naked embryos to being placed on water agar in the total absence of supporting nutrients (organic or inorganic). We note as well that in our laboratory all species tested have responded with improved vigor when nutrients were supplied. High vigor is very important to the successful germination of natural seed in agricultural soils. If vigor is reduced either by seed quality factors or weather, then problems with soil microbes will increase. This will also be true for manufactured seed.

7.3 *Manufactured Seed as Analogs of Botanic Seed*

Manufactured seed have improved in our laboratory as they have become functionally more like natural seed. This has involved improving vigor through providing an adequate oxygen supply and by creating a cotyledon restraint system that allows nutrients to be available at the moist embryo-interface surface. The cotyledon restraint system also channels the forces of embryo growth to facilitate timely extraction of the germinant from the seed. Further work is needed in this area to achieve the predictable 95% germination and rapid emergence from agricultural soil common to natural seed. We believe that manufactured seed should perform as well as natural seed in order to be commercially viable. They must be capable of withstanding broad temperature ranges and moisture conditions ranging from short-term flooding to short-term dry periods. They must also be biodegradable, and must have a constituency and vigor that will ward off infestation. Several of these requirements cannot be fully tested and developed until large scale field experiments can be implemented. Large scale field tests will not be practical until germination rates >80% in non-sterile soil freshly sampled from an agricultural field can be accomplished. Since traditional methods of methyl bromide/chloropicrin fumigation could no longer be available by the time of full implementation of this technology, seed should be designed to perform well in soil that has not been fumigated for several years. Seed must also be storable. Some storable desiccated seed designs have been proposed (Kitto and Janick 1980, 1986; Attree and Fowke 1993) but they are of more interest as methods of embryo desiccation than as seed since they require germination on nutrient media. Several tests have been performed with hydrated or partially hydrated gel encapsulation and cool storage (e.g. Lulsdorf et al. 1993; Redenbaugh and Fujii 1988). Pradhan et al. (2016) tested storage of Cymbidium Aliofolium (L) Sw. for up to 90 days. These authors found that viability was maintained at 83.3% when artificial seed were stored at 4 °C. In our labs, Manufactured seed have been stored for up to four months with no loss of

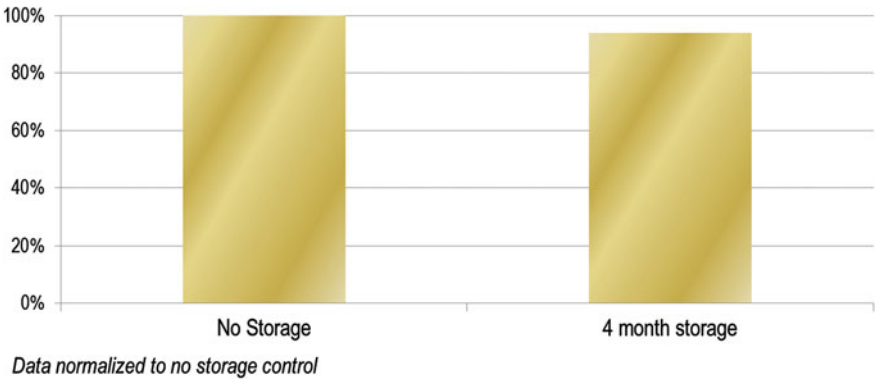

Fig. 5 Comparison of Weyerhaeuser Co. manufactured seed germination with and without four months cold storage. Manufactured seed stored for 16 weeks showed no significant loss of germination viability (ANOVA $p = 0.6387$)

germination vigor (Fig. 5). Optimally, manufactured seed should be storable for up to one year. This capability would allow maximum steady state manufacturing throughout the year with traditional crop sowing in the spring.

8 Conclusions

Manufactured seed technology has improved substantially. There is a high level of interest in the technology from agronomic, horticultural and forest industries. Manufactured seed technology could make it economically feasible to vegetatively propagate large numbers of plants originating as somatic embryos and sow the seed in agricultural fields with traditional equipment. This would be extremely valuable to several aspects of current genetics programs and could be critical to propagation of genetically engineered plants.

Both somatic embryo quality and manufactured seed design must improve prior to implementation of the technology. We believe that duplicating the chemical and physical attributes of natural seed that result in rapid emergence of normal germinants under field conditions will be the most productive way to approach research problems associated with operational use of manufactured seed.

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Somatic Embryogenesis in Scots Pine (*Pinus sylvestris* L.)

Malin Abrahamsson, David Clapham and Sara von Arnold

1 Introduction

Conifers are widely spread over the world and they have a considerable economical value as they are used in many countries as a source of pulp, timber, chemicals and fuel, and as ornamental plants (Raven et al. 1999). The coniferous trees Scots pine, *Pinus sylvestris* L., and Norway spruce, *Picea abies* (L.) Karst, are the most widely grown trees in the northern part of Europe. In Sweden, for instance, 55% of the land area is covered by productive forest land, of which 81% consists of Scots pine and Norway spruce (Swedish Forest Agency 2014).

A traditional conifer breeding program, including that for pine in Sweden, is a sophisticated but slow process because of the long generation time. Progenies from controlled crosses are grown in field trials at different locations. Owing to poor juvenile-mature correlations, selection with reasonable confidence of the best families for economically important growth traits is not possible until the trees are 10–15 years old. Material from selected trees is then taken for grafts and used in seed orchards for large-scale propagation; and in controlled crosses for the next cycle of breeding. In the seed orchards, much of the genetic gain from the breeding program is lost through sexual recombination and contamination with pollen from trees outside the orchards. Forest companies are therefore interested in the large-scale vegetative propagation of selected genotypes. Since it is hard to propagate Scots pine by cuttings, somatic embryogenesis is attractive as a possible alternative method. One proposed strategy is that embryogenic cell lines are established from the zygotic embryos from the controlled crosses in the breeding

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program (Högberg et al. 2003). A part of each cell line is cryopreserved (which enables waiting for the results of clonal testing) while plants are being regenerated from the other part of the culture. For several conifers both cryopreservation and the production of somatic embryo plants have been refined to the stage of implementation in practical forestry, including Multi-Varietal Forestry (MVF) described in Park et al. (2016).

Another approach to clonal mass propagation, at the same time integrating somatic embryogenesis into the traditional breeding program, is via 'family forestry' (Lindgren 2008): the mass propagation of untested individuals or clones from tested families, i.e. families that have already been tested in the course of the breeding program. Planting with a mixture of 10–30 clones from 5 to 6 tested families at each stand removes the dangers of excessive genetic uniformity associated with single clones, while exploiting the full genetic gains at the family level and avoiding the considerable expense and delay associated with effective clonal testing.

Another reason for interest in somatic embryogenesis in Scots pine is that somatic embryogenesis is a valuable model system for the study of embryogenesis, more convenient for experimentation than zygotic embryogenesis.

In an early report of somatic embryogenesis in Scots pine (Keinonen-Mettälä et al. 1996), the initiation frequencies varied between 0.2 and 9.0%, very few cell lines responded to maturation conditions, and few embryos were able to germinate. Since then, initiation frequencies of 20–30% have been obtained for a few selected genotypes, and with a very strict quality control of the mature embryos, good germination frequencies have been reported (Park et al. 2006; Lelu-Walter et al. 2008; Aronen et al. 2009). However, in Scots pine there are still overall problems with too low initiation frequencies, a high frequency of cell lines giving rise to abnormal somatic embryos, and poor germination of the mature embryos. The protocols presented in this chapter were used to carry out fundamental research in Scots pine embryogenesis (Abrahamsson et al. 2012, 2017); and the resulting knowledge will be valuable to improve culture conditions for propagating *Pinus* species on a large scale via somatic embryos. In addition we have performed a transcriptome analysis during early zygotic embryo development in Scots pine in order to pinpoint molecular processes regulating cleavage polyembryony (Merino et al. 2016).

The regeneration procedure includes: (i) initiation of embryogenic culture from whole megagametophytes containing immature zygotic embryos, (ii) proliferation of embryogenic tissue, (iii) pre-maturation for stimulating differentiation of early embryos, (vi) maturation of somatic embryos, (v) partial desiccation and (vi) embryo germination.

2 Culture Media Composition

DCR medium (Gupta and Durzan 1985), modified as previously described by Burg et al. (2007), is used as basal medium. The composition of the modified DCR medium is shown in Table 1.

Table 1 Composition of culture media for Scots pine

Ingredients	Initiation/ proliferation medium	Pre-maturation medium	Maturation medium	Germination medium
Minerals				
Macroelements (mg l^{-1})				
KNO_3	1491.5	1491.5	1491.5	373
NH_4NO_3	132	132	132	33
$MgSO_4 \cdot 7H_2O$	370	370	370	92.5
KH_2PO_4	170	170	170	42.5
$CaCl_2 \cdot 2H_2O$	170	170	170	170
Minerals				
Microelements (mg l^{-1})				
Kl	0.83	0.83	0.83	0.2075
H_3BO_3	6.2	6.2	6.2	1.55
$MnSO_4 \cdot 7H_20$	22.3	22.3	22.3	5.575
$ZnSO_4 \cdot 7H_20$	8.6	8.6	8.6	2.15
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25	0.0625
$CuSO_4 \cdot 5H_2O$	0.25	0.25	0.25	0.0625
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025	0.00625
$NiCl_2 \cdot 6H_2O$	0.025	0.025	0.025	0.00625
FeNaEDTA	6.95	6.95	6.95	1.7375
Vitamins (mg l^{-1})				
Myo-Inositol	200	200	200	50
Thiamine-HCL	2	2	2	0.5
Pyridoxine-HCL (B6)	0.5	0.5	0.5	0.125
Nicotinic acid	0.5	0.5	0.5	0.125
Amino acids (mg l^{-1})				
Glycine	2	2	2	0.5
Casein hydrolysate	500	500	500	125
L-Glutamine	450	450	450	112.5
Other compounds (g l^{-1})				
Sucrose	10	10		20
Maltose	–	–	30	–
PEG 4000	–	–	75	–
Gelrite[a]	3.5	3.5	3.5	3.5
Cefotaxime[b]	0.25	–	–	–
Hormones (μM)				
2,4-D	9	–	–	–
BA	4.4			
ABA	–	–	60	–

Modified DCR medium (Gupta and Durzan 1985), as previously described by Burg et al. (2007).
[a]For solidified medium. [b]For initiation medium

1. Add the plant growth regulators (PGRs) 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BA) before autoclaving.
2. Adjust the pH of the media to 5.8 ± 0.05 with 0.1 M NaOH or with 0.1 M HCL.
3. Solidify media with 0.35% (w/v) gellan gum (Gelrite®, Kelko, Atlanta, GA).
4. Sterilize media by autoclaving for 20 min.
5. Let the media cool down to approximately 58 °C, add filter sterilized glutamine and abscisic acid (ABA), and cast the Petri dishes (92 mm in diameter).

3 Initiation of Embryogenic Cultures

Embryogenic tissue of most conifers is initiated from immature or mature zygotic embryos placed on medium containing plant growth regulators. In *Pinus* species, together with other species that possess cleavage polyembryony, the most common way to initiate embryogenic tissue is from immature zygotic embryos before differentiation of cotyledons. When initiating from the very sensitive immature zygotic embryos, whole megagametophytes are often used as explants.

In Scots pine, it has so far only been possible to initiate embryogenic tissue from isolated megagametophytes containing embryos at the time just before or during cleavage polyembryony (Keinonen-Mettälä et al. 1996; Häggman et al. 1999; Lelu-Walter et al. 1999; Burg et al. 2007; Abrahamsson et al. 2017). This responsive period lasts only for approximately two weeks, and the start of cleavage polyembryony also varies to some extent from year to year. To ensure that the right developmental stage of the zygotic embryos is used for initiation, continuous spot-checks are strongly recommended, where the zygotic embryos are examined under the microscope to determine the developmental stage. The megagametophytes with embryos should preferable be excised and placed on initiation medium as soon as possible; however, cones can be stored at 5 °C for up to two months without affecting the potential to initiate embryogenic tissue (Häggman et al. 1999).

1. Surface sterilize the cones in 70% (*v/v*) ethanol for one to two minutes, and thereafter in diluted commercial bleach (e.g. Klorin original, Colgate-Palmolive, New York, NY) corresponding to about 0.5% sodium hypochlorite supplemented with a few drops of Tween® 20 for 20 min.
2. Rinse the cones abundantly with sterile distilled water and place them in sterile empty Petri dishes.
3. Open the cones using sterile scalpels and forceps, and isolate the immature seeds.
4. Excise the megagametophytes with embryos from the seeds and place them horizontally on solidified initiation medium.
5. Incubate the megagametophytes in darkness at 22 ± 1 °C. Embryogenic tissue starts to protrude from the micropylar end after two to seven weeks (Fig. 1a). After a few additional weeks, the small amount of embryogenic tissue should be separated from the megagametophyte and placed on a new plate containing proliferation medium.

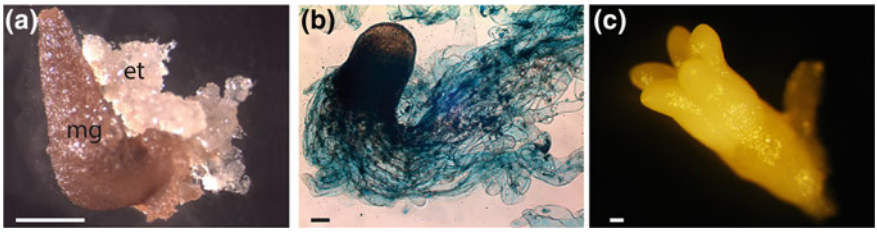

Fig. 1 Various developmental stages of somatic embryos in Scots pine. **a** Embryogenic tissue protruding from megagametophyte. **b** A late embryo, stained with Evans Blue, after 4 weeks exposure to ABA. **c** A cotyledonary embryo after 7 weeks exposure to ABA. *et* embryogenic tissue, *mg* megagametophyte. Bars **a** 1 mm, **b** 100 μm, and **c** 250 μm

4 Maintenance and Proliferation of Embryogenic Cultures

Embryogenic cultures of Scots pine are either proliferated on solidified medium, or as suspension cultures in liquid medium. For maintenance of embryogenic cultures, proliferation on solidified medium is the best and most frequently used method. The embryogenic cultures can usually be maintained for a year on solidified medium without any reduction in the quality of the culture. Embryogenic cultures in suspension have a higher proliferation rate than cultures on solidified medium, but tend to deteriorate more rapidly.

4.1 Sub-culture of Embryogenic Tissue Cultured on Solidified Medium

Embryogenic cultures are proliferated in 92 mm Petri dishes containing 20 ml solidified proliferation medium.

1. Transfer nine pieces (in total ca. 50 mg fresh weight) of white, translucent, embryogenic tissue (the outer region of the clump) to fresh proliferation medium.
2. The cultures are incubated in darkness at 22 ± 1 °C and sub-cultured every second week.

4.2 To Make a Cell Suspension Culture

1. Transfer four pieces (approximately 10 mm in diameter) of embryogenic tissue cultured on solidified medium into a 500 ml Erlenmeyer flask.

2. Add 100 ml of liquid proliferation medium and shake the flask carefully until the four pieces are finely divided.
3. If there is no air vent in the cap of the Erlenmeyer flask, do not close the cap too tight.
4. Place the flask on a gyratory shaker (125 rpm) in darkness at 22 ± 1 °C.

4.3 Sub-culture of Embryogenic Tissue Cultured in Suspension

1. Pour the suspension culture into 50 ml Falcon tubes. Let the tissue settle (you should preferably obtain 5–10 ml of settled tissue per Falcon tube).
2. Pour off the old medium carefully until tissue starts to follow. All the old medium does not have to be poured off.
3. Fill up the Falcon tubes with fresh proliferation medium and pour into a 500 ml Erlenmeyer flask. The final volume of proliferation medium should be 100 ml.
4. The cultures are incubated on a gyratory shaker (125 rpm) in darkness at 22 ± 1 °C and sub-cultured once a week.

5 Embryo Development and Maturation

To stimulate differentiation of early embryos and development of late (Fig. 1b) and cotyledonary embryos (Fig. 1c), the cultures are first transferred to liquid or solidified pre-maturation medium lacking PGRs for two to three weeks and then to solidified maturation medium containing ABA. In order to increase the osmotic potential, polyethylene glycol (PEG 4000) is added to the maturation medium. It takes 8–10 weeks before the embryos are fully mature (Abrahamsson et al. 2012) and ready for partial desiccation.

5.1 Embryogenic Tissue Cultured on Solidified Medium

1. Transfer five pieces (in total ca. 40 mg fresh weight) of white, translucent, embryogenic tissue (the outer region of the clump) to a 92 mm Petri dish containing 20 ml solidified pre-maturation medium.
2. After two to three weeks of incubation in darkness at 22 ± 1 °C, transfer the five pieces to a Petri dish containing fresh maturation medium.
3. The cultures are incubated in darkness at 22 ± 1 °C and sub-cultured every second week.

5.2 Embryogenic Tissue Cultured in Suspension

1. Pour the suspension culture into 50 ml Falcon tubes. Let the tissue settle (you should preferably obtain 10–15 ml of settled tissue per Falcon tube).
2. Pour off the old medium carefully until tissue starts to follow.
3. Fill up the Falcon tubes with fresh pre-maturation medium and pour into a 500 ml Erlenmeyer flask. The final volume of pre-maturation medium should be 100 ml.
4. The cultures are incubated for two to three weeks on a gyratory shaker (125 rpm) in darkness at 22 ± 1 °C and sub-cultured once a week.
5. Autoclave circular filter papers with a diameter of 70 mm (Whatman® qualitative filter paper, Grade 5) and a pile of paper tissues wrapped in aluminum foil.
6. Pour the suspension culture into 50 ml Falcon tubes and let the tissue settle.
7. Pour off the old medium carefully until the tissue starts to follow.
8. Place the sterile filter paper on the pile of sterile paper tissues (on the aluminum foil).
9. Pour the suspension culture in thin layers onto the filter paper.
10. Transfer the filter paper with tissue to a 92 mm Petri dish containing 20 ml maturation medium.
11. The cultures are incubated in darkness at 22 ± 1 °C and sub-cultured every second week.

6 Embryo Desiccation and Germination

In Norway spruce, the germination frequency is increased by the partial desiccation (desiccation at high humidity) of the embryos before germination (see von Arnold and Clapham 2008). It is unclear whether this is advantageous in pine species. It was used e.g. for radiata pine (*Pinus radiata* D. Don) but not for loblolly pine (*Pinus taeda* L.) and eastern white pine (*Pinus strobus* L.) (Klimaszewska et al. 2007). In Scots pine, partial desiccation was not used by e.g. Keinonen-Mettälä et al. (1996) and Aronen et al. (2009).

6.1 Partial Desiccation of Somatic Embryos

1. Place the lid of a 60 mm petri dish with the inside up in a 92 mm Petri dish. Pour approximately 3 ml of sterile water into the 92 mm Petri dish.
2. Transfer mature cotyledonary embryos from the maturation medium onto the lid of the 60 mm Petri dish. Seal the plate after transferring approximately 50 embryos.
3. The desiccating embryos are incubated in darkness at 22 ± 1 °C for three weeks.

6.2 Germination of Somatic Embryos

1. After the three weeks of desiccation, place the embryos horizontally on solidified germination medium in 150 mm petri dishes.
2. Plates with embryos are initially incubated at 22 ± 1 °C, either in darkness or under red light as was shown to be advantageous for somatic embryos of Norway spruce (Kvaalen and Appelgren 1999).
3. After about three weeks in darkness or under red light the germinating embryos are moved to white light at low intensity (10–20 µmol m^{-2} s^{-1}).
4. After one week of low intensity white light the intensity is increased to 100–200 µmol m^{-2} s^{-1}.

7 Cryopreservation

We are cryopreserving embryogenic cell lines of Scots pine following the protocol originally devised for embryogenic cultures of Norway spruce (Nörgaard et al. 1993; von Arnold and Clapham 2008), using a controlled-temperature cooling machine. The method we use is presented below. However, other methods have successfully been used to cryopreserve embryogenic tissue of Scots pine (Lelu-Walter et al. 2008).

7.1 Pre-treatment

1. Day 1. Transfer about 3 g of rapidly proliferating embryogenic tissue, sampled one week after subculture, to a sterile 100–150 ml container with lid (such as a baby food jar) containing 40 ml pre-maturation medium. Add 2 ml of a 4 M sorbitol solution in small amounts over 30 min. The suspension is incubated on a rotary shaker at 100 rpm in darkness at 22 °C for 24 h.
2. Day 2. Add sorbitol (4 M solution) to the suspension in small amounts to give a final concentration of 0.4 M sorbitol and incubate the cultures under the same conditions as during day 1for a further 24 h.
3. Day 3. Place the container with the suspension on ice for 15 min, together with 2 ml cryovials.
4. Add dimethyl sulfoxide (DMSO) in small amounts over 30 min to give a final concentration of 5% DMSO.
5. Disperse 1.0–1.5 ml settled tissue into each 2 ml cryovial and place on ice.

7.2 Cooling Program

Transfer the cryovials to a programmable controlled-temperature cooling chamber. The embryogenic tissue is frozen at a rate of 0.3 °C/min down to −16 °C and held at that temperature for 15 min. Then the tissue is frozen at 0.3 °C/min down to the final temperature of −35.5 °C, after which the cryovials are immediately transferred to a container filled with liquid nitrogen. The cultures are stored in liquid nitrogen until thawing.

7.3 Thawing

1. Move the cryotubes directly from liquid nitrogen to a container with water at 45 °C for approximately one minute or until the contents of the tube have just melted.
2. Move the tubes quickly to a container with sterile water at 4 °C for two to three minutes.
3. Surface sterilize the cryotubes in 70% (v/v) ethanol for two to three minutes.
4. Place the cryotubes standing in a sterile hood until all the ethanol has evaporated.
5. Open the lid of the cryotubes (without touching the area between tube and lid), and quickly pour out the cells onto an autoclaved circular filter paper with a diameter of 70 mm (Whatman® qualitative filter paper, Grade 5) placed on solidified proliferation medium.
6. Incubate the newly thawed cultures in darkness at 22 ± 1 °C (the Petri dishes should preferable be slightly tilted to make all the liquid drain from the filter paper). Transfer the filter paper to fresh proliferation medium after one hour, after one day, and if the culture still looks wet, after three days.
7. Embryogenic tissue should start to proliferate after approximately two weeks.

8 Protocol Optimization

The protocols for plant regeneration via somatic embryogenesis in Scots pine described in this chapter work adequately for experimental studies of a few cell lines. For commercial needs much optimization is necessary: (1) increased initiation frequency of embryogenic cultures, (2) increased frequency of embryogenic cell lines giving rise to cotyledonary embryos with normal morphology, (3) improved somatic embryo germination.

Initiation frequency from immature zygotic embryos depends greatly on the developmental stage of the embryos and the genotype of the parents (discussed in Aronen et al. 2009) and reported frequencies vary over the range 0–42%. Lowering

the concentrations of 2,4-D and BA raised the frequency of initiation in self- and cross-pollinated seed families to give rates of 3–25% (Lelu-Walter et al. 2008). The frequency of embryogenic cell lines of Scots pine giving rise to normal cotyledonary embryos is usually very low, e.g. 3.4% of initiated cell lines (Burg et al. 2007; von Arnold, unpublished). Furthermore, it has been suggested that most of the embryogenic cultures established from zygotic embryos at the stage of cleavage polyembryony will be abnormal and give rise to abnormal cotyledonary embryos (Abrahamsson et al. 2017). Even so, it may prove better to initiate embryogenic cultures from more differentiated tissues. To accomplish this, further research is needed to elucidate how to regain embryogenic potential in more mature tissues.

More extensive work on optimizing protocols is reported for other *Pinus* species including *P. taeda* and *P. radiata*, than for *P. sylvestris*, providing hints on what to investigate for *P. sylvestris.* The frequency of initiation in *P. taeda* has e.g. been raised several percentage units by including D-xylose and further raised by including D-*chloro*-inositol (Pullman et al. 2008) in the medium; and also raised by using liquid overlays (Pullman and Skryabina 2007). While PEG in the maturation medium improves the number of mature embryos in Norway spruce, it worsens the subsequent germination and growth of plantlets (Bozhkov and von Arnold 1998) and it is noteworthy that PEG is not included in the maturation medium for *P. radiata* where SE is showing commercial promise (Walter et al. 2005).

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Quercus Ilex L.

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1 Introduction

Holm oak (*Quercus ilex* L.) is the predominant tree species in many natural communities of the West Mediterranean. The dominant structure in the natural areas of this species is as wooded meadows, and its main economic importance is the agroforestry system. This structure is well known in Mediterranean countries and receives specific names, such as "dehesa" in Spanish, "montado" in Portuguese, etc. This agroforestry system is oriented to livestock production (mainly beef and Iberian pork) and hunting. Holm oak trees provide acorns as quality food for livestock and game, and sometimes for symbiotic cultures of truffle, which are becoming of high economic importance. Nevertheless, basic research on this species has been scarce. Problems are the variability of acorn production, the difficulty of conserving seeds and the lack of conventional vegetative propagation, especially for adult elite trees (Cornu et al. 1977; L'Helgoual'ch and Espagnac 1987). As holm oak has a prolonged life and a late sexual maturation with irregular reproductive cycles, difficulties are found for seed conservation, vegetative reproduction, and for the establishment of seed orchards. All these problems hamper the production of plant material for forest restoration, reforestation and for breeding and genetic

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programs. Furthermore, over the last years an intense dieback of holm oak has been recorded in southwest Spain, with *Phytophthora cinnamomi* and water stress believed to be the major factors involved (Romero et al. 2007; Solla et al. 2009; Corcobado et al. 2010).

In this scenario biotechnology approaches play a fundamental role for breeding and conservation purposes for this species. Micropropagation of oaks has been studied using apical and axillary bud cultures of *Q. ilex* juvenile cultures (Bellarosa 1989), and somatic embryogenesis of *Q. ilex* by culturing leaf explants (Féraud-Keller and Espagnac 1989) and immature zygotic embryos (Mauri and Manzanera 2003, 2004, 2005, 2011). For a recent review on the embryogenesis in oaks, see Gomez-Garay et al. (2014).

The potential in haploid research is now being realized with real and tangible results. In the past, doubled haploid has been restrained by technological problems but many hurdles have been overcome empirically and have resulted in major steps forward in the study of embryogenesis. A better understanding of embryogenesis in woody plants (i.e. Lippert et al. 2005; Sghaier-Hammami et al. 2009) and specifically on microspore embryogenesis (Gómez et al. 2009) should lead to more efficient protocols for the production of haploid and doubled haploid plants. The induction of microspore embryogenesis could be obtained by different systems of anther and microspore cultures. In general, the stress inductive treatment generates cellular responses and plays a major role in microspore embryogenesis. Heat or cold stress treatments are commonly used for anther or microspore culture of some species such as *Quercus suber* (Bueno et al. 1997), *Q. ilex* (Pintos et al. 2013) or *Populus nigra* (Deutsch et al. 2004). It has been proposed that cold treatment slows the degradation process in anther tissue, thus protecting microspores from toxic compounds released in decaying anthers and so assures the survival of a greater portion of embryogenic pollen grains as compared to heat treatment (Duncan and Heberle 1976).

Production of doubled haploid (DH) comprises two main steps: induction of microspore embryogenesis and duplication of the haploid genome. Such duplication is sometimes indirectly induced by the treatments used to promote sporophytic development. Thus, low temperature also increases the frequency of endo-reduplication leading to an increase of spontaneously doubled-haploid plants (Amssa et al. 1980). Nevertheless, an additional step of direct chromosome doubling usually should be included in the protocol (Pintos et al. 2007).

The totipotency of plant cells favours the regeneration from either haploid cells of the anther cavity (microspores) or from diploid cells of the anther wall. In this sense, flow-cytometry can be used to analyse the ploidy level of the embryos (Bueno et al. 2003). Furthermore, a genetic test was designed through microsatellite markers to elucidate if diploid embryos were originally haloids which spontaneously duplicated their genome, or alternatively those embryos were regenerated from the diploid tissue of the anther wall (Gomez et al. 2001). Thus, the breeding process of *Q. ilex* has been shortened through the induction of haploid embryos and doubled-haploids (Pintos et al. 2013).

2 Materials

All media must be prepared using distilled water and cell culture grade reagents. Reagents must be stored at room temperature (unless indicated otherwise). Disposal regulations must be followed for waste materials.

Induction medium

1. Basal medium (SM) containing (Sommer et al. 1975) and microminerals and cofactors (Murashige and Skoog 1962).
2. 3% (w/v) sucrose.
3. 1% (w/v) activated charcoal.
4. 0.8% (w/v) agar.
5. Adjust pH to 5.6.
6. The medium must be autoclaved at 0.1 MPa (121 °C) for 20 min.
7. When culture medium reaches 60 ± 5 °C dispose it in Petri dishes (12 cm diameter) in a laminar flow hood until agar jellification.
8. Preserve media-plates in sealed plastic bags at 4 ± 1 °C until use.

Proliferation medium

1. Basal medium (SM) containing (Sommer et al. 1975) and microminerals and cofactors (Murashige and Skoog 1962).
2. 500 mg/l glutamine.
3. 3% (w/v) sucrose.
4. 0.8% (w/v) agar.
5. Adjust pH to 5.6.
6. The medium must be autoclaved at 0.1 MPa (121 °C) for 20 min.
7. When culture medium reaches 60 ± 5 °C dispose it in Petri dishes (12 cm diameter) in a laminar flow hood until agar jellification.
8. Preserve media-plates in sealed plastic bags at 4 ± 1 °C until use.

Maturation medium

1. Basal medium (SM) containing (Sommer et al. 1975) and microminerals and cofactors (Murashige and Skoog 1962).
2. 1% activated charcoal.
3. 3% (w/v) sucrose.
4. 0.8% (w/v) agar.
5. Adjust pH to 5.6.
6. The medium must be autoclaved at 0.1 MPa (121 °C) for 20 min.
7. When culture medium reaches 60 ± 5 °C dispose it in Petri dishes (12 cm diameter) in a laminar flow hood until agar jellification.
8. Preserve media-plates in sealed plastic bags at 4 ± 1 °C until use.

Germination medium

1. Basal medium (SM) containing (Sommer et al. 1975) and microminerals and cofactors (Murashige and Skoog 1962).
2. 0.05 mg/l 6-benzylaminopurine (BA)
3. 0.1 mg/l indole-3-butyric acid (IBA).
4. 1.5% (w/v) sucrose.
5. 1% (w/v) agar.
6. Adjust pH to 5.6.
7. The medium must be autoclaved at 0.1 MPa (121 °C) for 20 min.
8. When culture medium reaches 60 ± 5 °C dispose it in Petri dishes (12 cm diameter) in a laminar flow hood until agar jellification.
9. Preserve media-plates in sealed plastic bags at 4 ± 1 °C until use.

Flow cytometry

Extraction buffer and staining solution (DAPI): Partec Cystain UV precise P Kit.

Solutions for DNA extraction

1. 1 M Tris-HCl, pH 8 and 1 M Tris-HCl, pH 7.5: Tris (hydroxymethyl) aminomethane (FW 121.4 g/mol). Weigh 60.57 g Tris in 0.5 l water. Mix and adjust adequate pH with HCl. Store at 4 °C.
2. 3.8 g/l Sodium bisulfite (sodium hydrogen sulfite): weigh 3.8 g sodium bisulfite and make up to 1 l water.
3. Extraction buffer 1, pH 8: 0.35 M Sorbitol (FW 182.17 g/mol), 0.10 M Tris-HCl (pH 8), 5 mM EDTA (Ethylenediaminetetraacetic acid, FW 372.24 g/mol). Weigh 15.94 g Sorbitol and 0.47 g EDTA. Transfer to 25 ml 1 M Tris-HCl (pH 8) from previous step and make up to 250 ml with water. Mix and adjust pH with HCl or NaOH.
4. Lysis buffer, pH 7.5: 200 mM Tris (pH 7.5), 50 mM EDTA (Ethylenediaminetetraacetic acid, FW 372.24 g/mol), 2 M NaCl (FW 58.44 g/mol), 20 g/l CTAB (Cetyl trimethylammonium bromide, FW 364.45). Weigh 4.7 g EDTA, 29.22 g NaCl and 5 g CTAB. Transfer to 50 ml 1 M Tris-HCl (pH 7.5) from step 1 and make up to 250 ml with water. Mix and adjust pH with HCl or NaOH.
5. 5% Sarkosyl: 50 g/l N-laurylsarcosine (FW 293.38). Weigh 25 g N-laurylsarcosine and make up to 0.5 l with water, mix.
6. TE buffer, pH 8: 10 mM Tris-HCl (pH 8) and 1 mM EDTA (Ethylenediaminetetraacetic acid, FW 372.24 g/mol). Weigh 0.37 g EDTA. Transfer to 10 ml Tris-HCl (pH 8) from step 1 and make up to 1 l with water. Mix and adjust pH with HCl or NaOH. EDTA will not be soluble until pH reaches 8.0.
7. 70 % Ethanol.

Chemicals for Simple Sequence Repeat (SSR) amplifications

1. Taq-DNA polymerase with the buffer supplied for Taq-DNA polymerase amplification, (including Tris-HCl pH 9, KCl, $MgCl_2$).
2. dNTPs (dATP, dCTP, dGTP and dTTP).
3. Fluoro-labelled oligonucleotide primers. The forward primer of each pair (see Table 1) was labelled with fluorescent dye (i.e.: FAM, HEX and TET) to allow detection of the polymerase chain reaction (PCR) products by an automatic DNA-sequencer.

3 Methods

Induction of microspore embryogenesis

The induction of microspore embryogenesis of holm oak is obtained from anther cultures. During the process, the embryogenic response is supervised at three structural levels: catkins, anthers and microspores, and the parallel development thereof. In addition, we describe the influence of the chilling pre-treatment (4 °C) on the anther response and the heat shock stress treatments.

Correlation between the developmental stages of catkins, anthers and microspores

Catkins are collected during middle flowering at different phenologic stages, finding a relationship with the anther phenology (Fig. 1a). Anthers from those stages are squashed and the respective microspores are stained with 4′-6-diamidino-2-phenylindole (DAPI) for the determination of their developmental stage (Fig. 1b–d). The highest rate of late vacuolated microspores and early bicellular pollen grains was found in 1–1.5 cm long, green-yellow anthers. Late microspores show a polar nucleus and a great central vacuole. These highly vacuolated late microspores are the optimal phase for induction of microspore embryogenesis (Fig. 1c).

Table 1 Characteristics of the microsatellite loci amplified in *Quercus ilex*

Locus	Repeat motif	Primer sequences (5′–3′)	Allele size range	Annealing temp (°C)
QpZAG36	$(GA)_{23}$	gatcaaaatttggaatattaagagag actgtggtggtgagtctaacatgtag	211–225	50
QrZAG20	$(TC)_{18}$	ccattaaaagaagcagtattttgt gcaacactcagcctatatctagaa	162–170	52

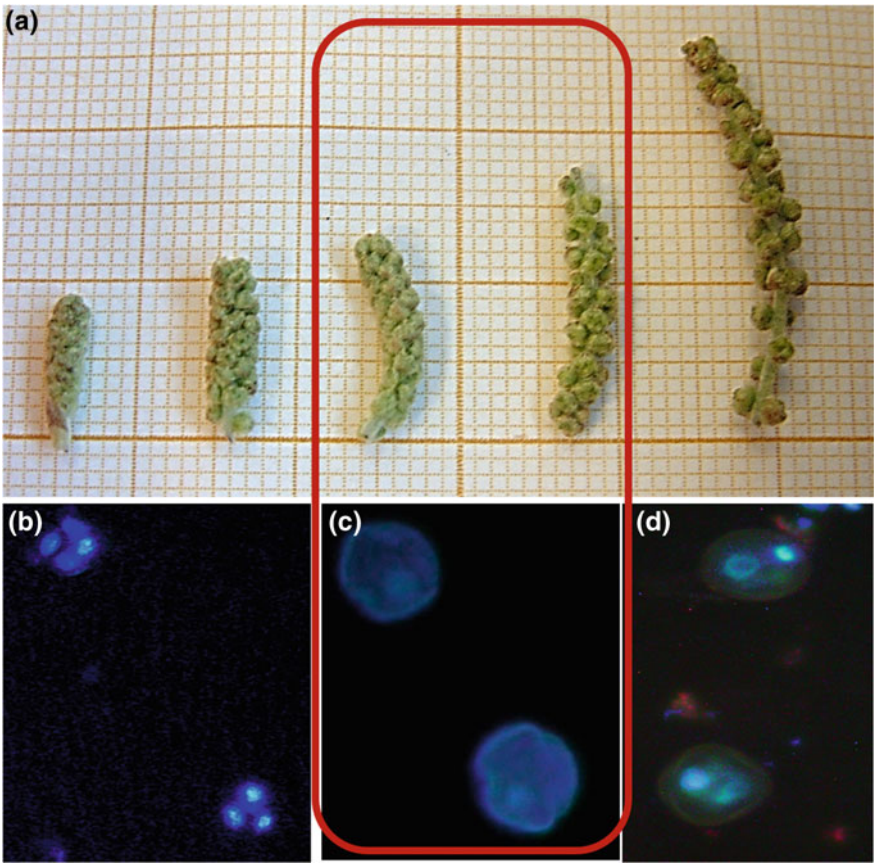

Fig. 1 Characterisation of the parallel development of catkins and microspores before pollen release in Quercus ilex L. **a** Developmental stages of catkins. **b** Microspores at the tetrad stage. **c** Microspores at the late uni-nucleated or vacuolated stages. **d** Early bi-nucleated pollen stage. red rectangle indicates the optimum stage for microspore embryogenesis

Surface sterilization procedure

Catkins are surface-sterilized by immersion in 70% ethanol for 30 s and then placed in 2% sodium hypochlorite solution with a few drops of Tween 20 for 20 min, followed by three rinses in distilled sterile water.

Cold stress pre-treatment

Around 40 anthers with vacuolated late microspores are sowed per plate in the induction medium. Sowed anthers were subjected to a cold stress pre-treatment of 4 ± 1 °C for 3–5 days in the dark.

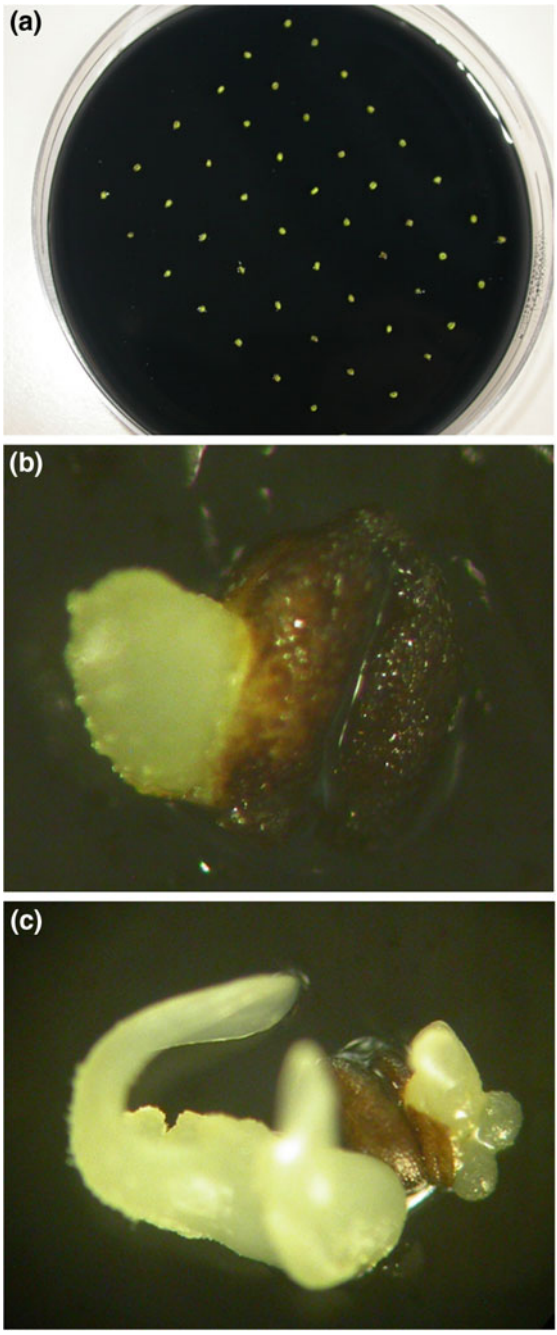

Fig. 2 Microspore embryogenesis induction from Quercus ilex L. anther cultures. **a** Holm oak anther cultures on induction medium. **b** Detail of small globular anther embryos emerging from inside the anther. **c** Globular and immature cotyledonary embryos formed by direct embryogenesis from an anther

Heat shock treatment

Petri dishes containing the anthers on the induction medium are transferred to an incubation chamber and are subjected to heat shock at 33 ± 1 °C for 2–5 days in the dark.

Anther culture

Plates with the anthers (Fig. 2a) are transferred to a tissue culture chamber at 22 ± 2 °C in the dark until the embryos emerge from the interior of the embryogenic anthers, breaking through the walls (Fig. 2b, c).

Embryogenic masses are transferred to proliferation medium and are maintained in the dark. Initial translucent globular embryos (Fig. 3a) will later develop into heart-shaped and torpedo-shaped embryos, until formation of well-developed cotyledons can be observed (Fig. 3b).

Maturation of *Quercus ilex* L. embryos

Cotyledonary embryos are transferred to maturation medium at 25 °C during 4 weeks, in the dark. Then, the plates containing the embryos are transferred to a cool chamber at 4 °C during two months. Mature embryos accumulate reserve nutrients and cotyledons largely increase their size (Fig. 4a).

Germination of *Quercus ilex* L. embryos

Mature embryos are prepared for the germination treatments by their hydration in sterile distilled water for 24 h at 4 °C. Then, they are transferred to germination medium and placed in a tissue culture chamber at 22 ± 2 °C with a 16 h light and 8 h darkness photoperiod (Fig. 4b).

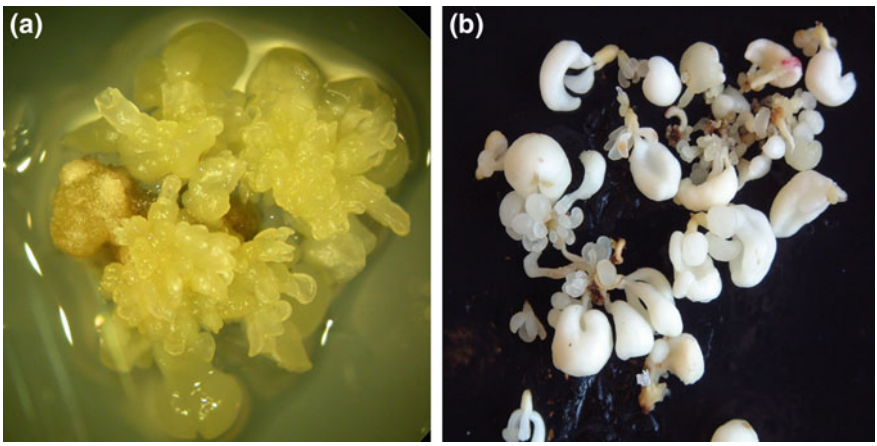

Fig. 3 Proliferation of holm oak haploid embryos. **a** Embryos at different developmental stages and clumps of embryogenic masses formed on proliferation medium. **b** Mature cotyledonary embryos culture on maturation medium

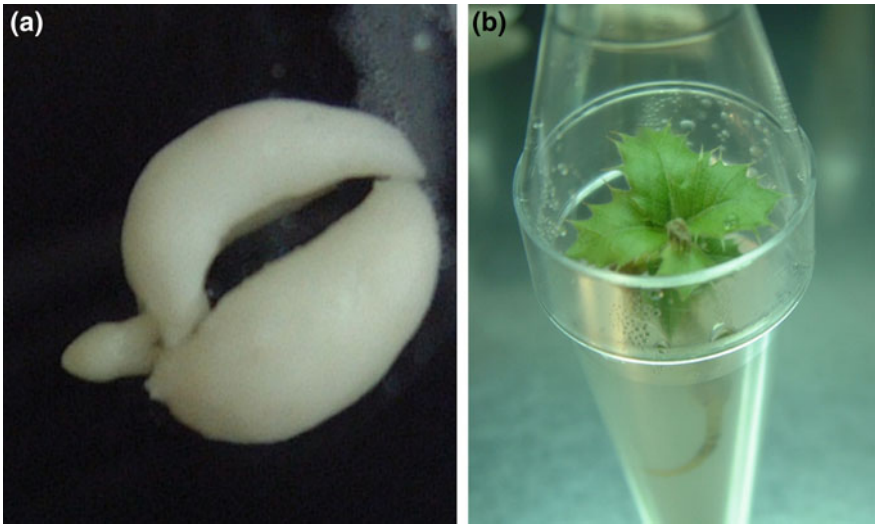

Fig. 4 Maturation and germination of holm oak haploid embryos. **a** Mature cotyledonary embryo before the germination process. **b** Holm oak plantlet derived from microspore embryogenesis

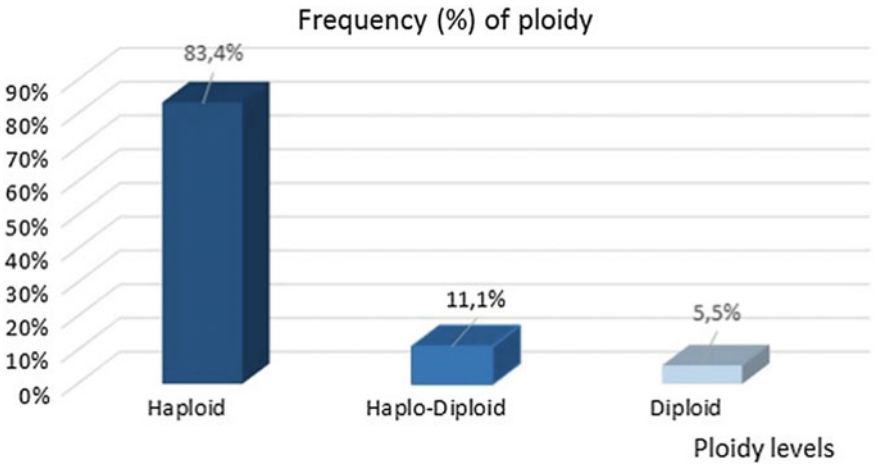

Fig. 5 Frequency (%) of different ploidy levels of anther-derived holm oak embryos

Flow cytometry analysis

The ploidy level is analysed by flow cytometry in anther-derived embryos (Fig. 5).

1. 0.5 cm^2-size embryogenic mass must be chopped with a sharp razor blade in a 55 mm plastic Petri dish containing 500 µl extraction buffer for nucleus release.

2. Filter it through a Partec 30 µm celltrics disposable filter.
3. The suspension of released nuclei is then stained with 1500 µl staining solution in a 2 ml eppendorf tube for 60 s.
4. The relative fluorescence of total DNA from isolated nuclei is analysed with a PA Ploidy Analyzer, Partec. Sample size must be was at least 10,000 nuclei. To determine the standard peak of diploid cells (2C DNA), diploid tissue from the parent *Q. ilex* leaves is used. 5. The standard peak is adjusted to channel 100 of relative fluorescence intensity (Pintos et al. 2013).

DNA extraction

1. Weight out 70 mg embryo tissue in a 2 ml microcentrifuge tube. Add 100 µl Sodium bisulfite (3.8 g/l).
2. Add 300 µl extraction buffer 1 and grind the embryo with a sterilized tip.
3. Add 300 µl lysis buffer. Mix gently.
4. Add 120 µl sarkosyl (5%) and mix vigorously.
5. Incubate for 15 min at 65 °C in a water bath.
6. Take the sample off the bath and add 600 µl chloroform. Cap the tube and mix vigorously by vortex to obtain an emulsion.
7. Centrifuge for 10 min at 12,000 rpm and 4 °C. Transfer the upper phase to a clean microcentrifuge tube and add 400 µl volume of −20° isopropyl alcohol and gently invert the tube several times to mix.
8. To precipitate the DNA, place the tube at −20 °C for 30 min.
9. To pellet the DNA, centrifuge the tubes at 12,000 rpm for 5 min at 4 °C.
10. Leave the pellet air-drying. Add a drop of 70 % ethanol to wash the pellet. Air-dry the pellet again.
11. Re-dissolve the pellet in 50 µl of TE pH 8 in the water bath for 15 min at 65 °C.
12. Store the DNA at −20 °C.

SSR amplifications by PCR

1. Preparing the reaction mixes: (see Table 2).

Table 2 Amplification reaction mixture in 25 µl final volume/concentration per reaction for *Q. ilex* SSR amplification

Step	Reagent	Final concentration
1	Taq-DNA polymerase	1.75 U
2	Tris-HCl (pH 9.0)	10 mM
3	KCl	50 mM
4	$MgCl_2$	1.5 mM
5	dNTPs	200 µM
6	Primer forward (1 µm)	200 nM
7	Primer reverse (1 µm)	200 nM
8	Sterile Milli-Q water	Up to 23 µl
9	DNA template (10 ng/µl)	2 µl

Table 3 Thermocycling profiles for amplification Of *Quercus ilex* SSRS

Locus	Amplification profiles
QpZAG36	95 °C, 6′/[92 °C, 1′/50 °C, 30″/72 °C, 1′] x 30/72 °C, 8′/4 °C, ∞
QrZAG20	95 °C, 6′/[92 °C, 1′/52 °C, 30″/72 °C, 1′] x 30/72 °C, 8′/4 °C, ∞

2. Running the PCR: PCR must be conducted in a PCR-thermocycler following the manufacturer's instructions and with cycling conditions listed in Table 3.

Analyzing the data

1. Both microsatellite markers are analyzed together by the automatic sequencer; each of them must have a different Dye in order to discriminate the results.
2. Peak sizes are quantified by comparison with internal size-standards using software provided by the automatic sequencer manufacturer.
3. The genotype of each sample (leaves from parent tree, haploid, diploid and doubled-haploid embryos) is defined as the combination of the analysed fragments (Fig. 6).

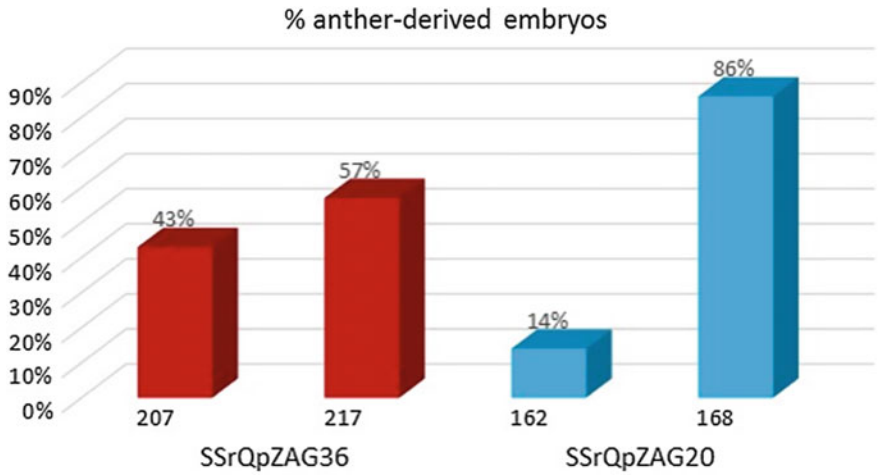

Fig. 6 Anther induced embryos bearing each microsatellite allele detected in holm oak parent tree

4 Conclusion and Future Prospects

The propagation method of *Quercus ilex* through anther embryogenesis is presented. Induction of haploid embryos and doubled haploids is obtained in *Q. ilex* by temperature stress treatments of anthers containing late vacuolated microspores because this developmental stage is responsive to embryogenesis induction in holm oak microspores. The embryos grow in the interior of the anthers, breaking through the degenerating anther walls. Under these optimal conditions, embryo formation occurs between 2.5 and 3 months after induction. The haploid origin of the anther embryos and/or spontaneous doubling of the chromosomes during early regeneration stages are confirmed by quantitative nuclear DNA analysis through flow cytometry and by DNA microsatellite markers. The production of haploids and DH through gametic embryogenesis provides an attractive biotechnological tool for developing homozygous lines from heterozygous parents, which is important in breeding programs, as well as in genetic studies. Furthermore, using doubled-haploid lines (DH), multiple disease phenotype datasets, it could be possible to determine the genes (and their effects) for conferring resistance to diseases.

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Hybrid Larch (*Larix* × *eurolepis* Henry)

Anna Kraft and Marianne Kadolsky

1 Introduction

The native range of European larch is small and scattered across Europe in comparison to other native conifers in Europe; the cultivated areas have been widely expanded by forestry (Pâques et al. 2013). The importance of *Larix spec*. has been further increased by hybrid larch (*Larix* × *eurolepis* Henry), which possesses economical relevant heterosis effects.

Larix × *eurolepis*, the hybrid between European larch (*Larix decidua* Mill.) and Japanese larch (*Larix kaempferi* (Lamb.) Carrière) was first described by Henry and Flood (1919). This conifer hybrid is of interest, due to its remarkable characteristics like fast growth, superior stem form and the mechanical wood properties and a wide range of plantation sites (Pâques 1989, 1992; Li and Wyckoff 1994; Larsson-Stern 2003; Pâques et al. 2009). Unfortunately, the hybrid seed yield is infrequent and unreliable (Lelu et al. 1994a), hence a lot of research on hybrid larch was initiated and followed through during the last decades (Klimaszewska et al. 2016). Research topics concern e.g. breeding strategies, cryopreservation and plant regeneration from embryogenic cultures, acclimatization of micropropagated shoots from mature material as well as studies on potential advantages over Norway spruce in commercial forestry in Sweden (Pâques 1989; Klimaszewska et al. 1992; Brassard et al. 1996; Larsson-Stern 2003; Ewald 2007).

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Furthermore a hybrid larch breeding program, running since the nineties, in the public enterprise "Staatsbetrieb Sachsenforst", Saxony, Germany is aiming at exploiting the advances in the field combining the clonal propagation technique and starting material from a superior genetic background (controlled crosses). For clonal mass propagation of conifers somatic embryogenesis is the method of choice to produce plants from selected parental premium crossing combinations (Zoglauer et al. 2003). To establish and improve the individual in vitro steps, several studies with various conifers have been conducted. In summary, the in vitro process of somatic embryogenesis is similar among most tested conifer species. Immature or mature zygotic embryos are the starting material for the initiation of somatic embryogenesis. These are cultured on induction medium containing auxins and/or cytocinins. The maturation of somatic embryos is initiated by using abscisic acid and polyethylene glycol as non-plasmolytic osmoticum (or other high molecular weight solutes), whereas the germination takes place on media without plant growth regulators, a high gel strength and activated charcoal (Klimaszewska et al. 2016). With regard to *Larix spec.*, several studies were conducted in the last decades, describing and analyzing the different steps of in vitro somatic embryogenesis (Klimaszewska 1989; Lelu et al. 1994b; Gutmann et al. 1996; Lelu-Walter and Pâques 2009). However, specific and detailed publications on acclimatization of *Larix* × *eurolepis* are scarce. Hence, new protocols were established based on long-standing experiences and general knowledge about the distinctive features of micropropagated plants (Donnelly and Tisdall 1993; George et al. 1993) such as the impaired ability to regulate water loss and the mixotrophic mode of nutrition.

Here, we describe a comprehensive protocol for initiation, maturation, germination and cryopreservation of in vitro somatic embryogenesis. Due to a high economical interest in this hybrid, close collaborations between public enterprises (Staatsbetrieb Sachsenforst) and research (Humboldt-Universität) have recently made advances in the field of acclimatization and plant production to complete the set of protocols.

2 Material

1. Immature seeds containing embryos at precotyledonary to cotyledonary stage
2. Laminar-flow hood, mercuric-chloride ($HgCl_2$), 50 ml tubes, forceps, scalpel, dissecting microscope, Petri dishes (60 mm), culture vessels (60 mm: maintenance and maturation, 85 mm: germination), Pasteur pipet (3 ml), paper filter (50 mm diameter), paper towels, cryopreservation tube (1.5 ml), liquid nitrogen
3. Growing room
4. Media (see Table 1)
5. Mini-Jiffys: Jiffy-7 Forestry Peat Pellets, 18 × 42 mm, in short trays, 256 caverns
6. QuickPot QP 35T trays, caverns 50 × 50 × 115 mm

Table 1 Hybrid larch basic culture media for in vitro processes (modified according to Becwar et al. 1990)

Chemicals	Induction/maintenance (MSG-1) [mg/l]	Maturation [mg/l]	Germination [mg/l]
KNO_3	100	100	–
KCl	745	745	–
KH_2PO_4	170	170	340
$CaCl_2 \cdot 2H_2O$	440	440	134
$MgSO_4 \cdot 7H_2O$	320	320	400
$Ca(NO_3)_2 \cdot 4H_2O$	–	–	220
$MnSO_4 \cdot H_2O$	16.9	16.9	20.8
H_3BO_3	6.2	6.2	5
$ZnSO_4 \cdot 7H_2O$	8.6	8.6	8
KI	0.83	0.83	1
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.2
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.024
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025
FeNaEDTA	36.7	36.7	36.7
Myo-inisitol	100	100	100
Nicotinic acid	0.5	0.5	0.5
Pyridoxin-HCl	0.5	0.5	0.5
Thiamin-HCl	1	1	1
L-glutamine	1460	1460	730
Glycin	–	–	2
2.4-D	2	–	–
ABA	–	10.5	–
6-BAP	0.5	–	–
Caseinhydrolysate	–	–	500
Sucrose	20,000	30,000	20,000
PEG 4000	–	100,000	–
Activated charcoal	–	–	1000
Gelrite	3300	3200	3000

7. Conifer container substrate, pH 4.5
8. Greenhouse with fogging chambers for controlled humidity.

The pH is adjusted to 5.8 with KOH or HCl before autoclaving at 121 °C for 15 min. ABA and L-glutamine are filter sterilized and added after autoclaving at a temperature of approximately 60 °C.

8 ml induction medium are filled in Petri dishes (60 mm), 25 ml maintenance or maturation medium in small culture dishes (60 mm) and 50 ml germination medium in large culture dishes (85 mm).

3 Method

The in vitro process chain consists of four steps: 1. initiation of embryogenic cultures, 2. maintenance and proliferation of somatic embryos, 3. maturation, 4. germination, followed by two ex vitro steps: 5. pricking seedlings out in substrate and acclimatization, 6. cultivation and hardening off of young plants. For cryopreservation for long-term storage use embryogenic culture (EC) that were freshly subcultered on maintenance medium and grown for 10 days.

Initiation of embryogenic cultures

Collect fertilized immature female cones in the period from end of May to July (depending on the climate conditions) and start the dissection of the seeds immediately (with a maximum storage period of one week at 4 °C).

1. Remove the seeds from the immature cones.
   Conduct the following steps in the laminar-flow hood:
2. Sterilize the seeds with 0.1% $HgCl_2$ for 3 min.
3. Wash the seeds with sterile distilled water for 3 min, 3 times.
4. Dissect the seed coat and remove the megagametophyte (Fig. 1).
5. Cut the megagametophyte laterally and place it on the surface of induction medium. Make sure that the cut surface touches the medium.
6. Incubate the explants in darkness at 23 °C for 6–8 weeks.
7. Check the sterility of the explants regularely and remove contaminated megagametophytes.

Maintenance and Proliferation of somatic embryos

1. Select the EC: Make sure to separate every potential induction event from callus proliferation by using the microscope (Fig. 2).
2. Transfer EC to MSG-1 in small culture dishes and incubate them in darkness at 23 °C.
3. Subculture the EC by transferring them to fresh medium every 4 weeks.

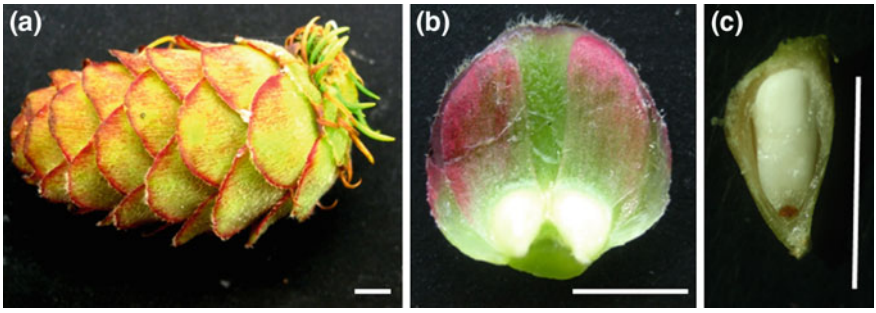

Fig. 1 Dissection of immature *Larix* × *eurolepis* seeds. **a** Immature female cone, bar 0.5 cm. **b** Covering scale with seed scale, bar 0.5 cm. **c** Laterally opened seed containing the megagametophyte, bar 0.5 cm

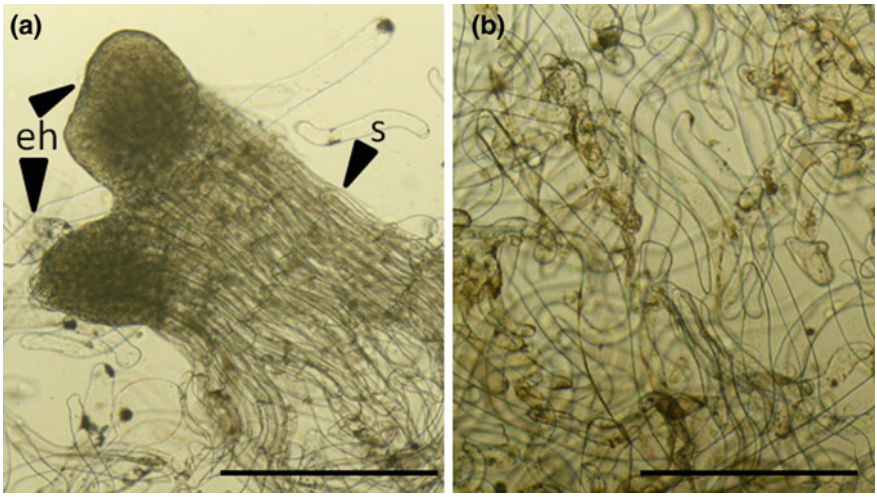

Fig. 2 Somatic embryos of *Larix* × *eurolepis* compared to callus cells. **a** Early stage somatic embryos with embryonal head (eh) and suspensor cells (s), 0.5 mm. **b** Callus cells, 0.5 mm

Cryopreservation

EC can be stored in liquid nitrogen for long term storage.

1. Collect 3 g EC from 10 day old proliferation cultures and cultivate on MSG-1, containing 0.4 M sorbitol for 24 h.
2. Remove liquid from EC on sterile paper towels and homogenize it with 9 ml liquid MSG-1, containing 0.4 M sorbitol and 12 ml liquid MSG-1, containing 0.4 M sorbitol and 10% DMSO.
3. Keep suspension on ice and under rotation for 30 min.
4. Fill 1.5 ml suspension into each cryopreservation tube and cool the samples down to −35 °C (0.3 °C/min).
5. Transfer the tubes into liquid nitrogen.
6. To thaw the somatic embryos, heat up the samples at 39 °C in a water bath for 2–3 min.
7. Add 9 ml liquid MSG-1, containing 0.4 M sorbitol and incubate it 30 min under rotation at room temperature.
8. Apply vacuum to remove the liquid and transfer the EC to MSG-1, containing 0.4 M sorbitol.
9. Transfer the EC to MSG-1, containing 0.2 M sorbitol after 24 h.
10. Transfer the EC to MSG-1 after 24 h.

Maturation of somatic embryos

The maturation of somatic embryos is initiated by a change of plant growth regulators and the addition of polyethylene glycol (PEG). Exogenous abscisic acid (ABA) prohibits early germination and leads to high quality embryos in large

quantities (Gutmann et al. 1996), while PEG causes osmotic stress, which stimulates maturation of somatic embryos (Arnold et al. 2002).

1. Select fresh EC from 11 day old proliferation cultures.
2. Remove remaining phytohormones by washing the cultures with sterile distilled water. Dry the somatic embryos on sterile paper towels.
3. Suspend the EC in sterile distilled water (100 mg/ml) (for an optimal blending, mix the suspension by thoroughly pipetting).
4. Distribute the suspension on paper filter (100 mg/filter), remove excessive liquid by placing the paper filter on top of sterile paper towels prior to transfer to maturation medium. Cultivate the EC for 5 weeks in darkness at 23 °C (Fig. 3a).

Germination of somatic embryos

To induce germination of mature somatic embryos, they are transferred to medium without phytohormones, which contains activated charcoal to absorb undesired substances like plant waste products and toxic metabolites and to provide a dark environment for improved rooting (Pan and Staden 1998).

1. Select morphologically intact, non-deformed mature embryos with proper root development and transfer them to germination medium (Fig. 3b).
2. Cultivate the embryos 3 days in darkness and 11 days in light (light for 16 h day length, 120 μE) (Fig. 3c).

Acclimatization and young plant culture

The transfer of plants from in vitro to ex vitro conditions means the adaption to a dramatically changed environment: Higher light intensity, lower air humidity, higher water potential of the substrate, higher rates of gas exchange, varying temperature, no carbohydrates in the medium. This adaption process requires careful accommodation to be successful and to result in an end product of high quality and economic value. The process requires the following steps: (1) Quality

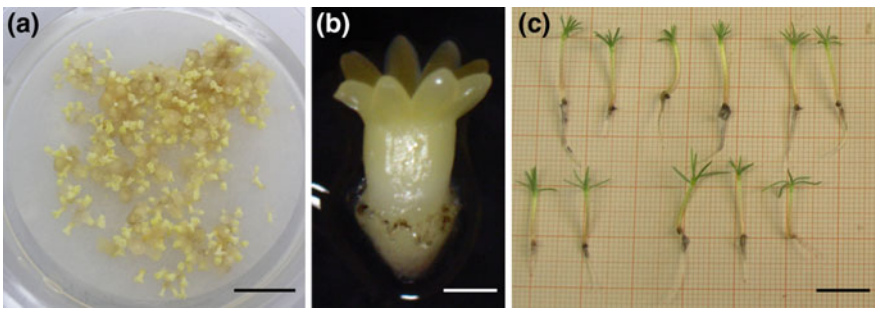

Fig. 3 Somatic embryos and seedlings of *Larix* × *eurolepis*. **a** Mature somatic embryos on maturation medium after 5 weeks of cultivation, 1 cm. **b** Mature somatic embryo, 1 mm. **c** Somatic seedlings after 3 days in darkness and 11 days in light on germination medium, 1 cm

sorting of somatic seedlings, (2) pricking out and acclimatization of plantlets, (3) cultivation and growth control of young plants.

1. Quality sorting of somatic seedlings: Take somatic seedlings out of the medium using forceps, place them on a paper towel and select those which meet all of the following criteria (Fig. 4a):

   – Shoots longer than 0.5 cm, of greenish-white color
   – First needles appear above the cotyledons, both of greenish color
   – Roots as long as shoots or longer, of white color
   – Healthy, normal appearance, absence of any signs of hyperhydricity or deterioration.

   Cut back any roots that are longer than 1.5 cm with a sharp scalpel.
   Remove any medium that might adhere to the seedlings.
   Make sure seedlings do not dry out during this process. Thus, collect them in an appropriate, wet paper towel lined container, until pricking out is about to start.

2. Pricking out and acclimatization of plantlets: In the greenhouse, only use well-watered Mini-Jiffys. Make a sufficiently deep and wide hole and place one plantlet per Mini-Jiffy. Make sure that root and stem are straight. Gently press the substrate around the plantlet (Fig. 4b). During the whole process, make sure the plantlets do not dry out. Place them in a greenhouse-chamber with the following conditions:

   – Ambient temperature between 20 °C (day) and 15 °C (night)
   – Air humidity close to 100%
   – Long day conditions: natural light supplemented with assimilation light for 16 h day length.

After two weeks, decrease humidity to 80%. After another two weeks discontinue to control humidity altogether. After one more fortnight acclimatization is completed. At this point a regular fertilization is recommended to ensure continuous growth and avoid the transition into dormancy (dormant bud formation).

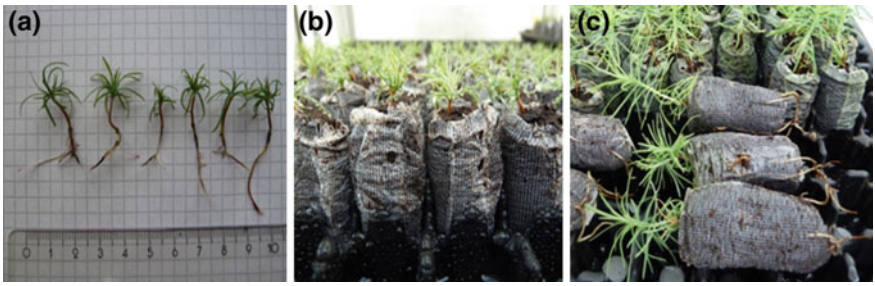

Fig. 4 Plantlets of *Larix* × *eurolepis* derived from somatic embryogenesis. **a** Well developed, first quality plantlets before being pricked out. **b** Freshly pricked out plantlets in Mini-Jiffy. **c** Acclimatized plantlets show roots on the surface of the Mini-Jiffy

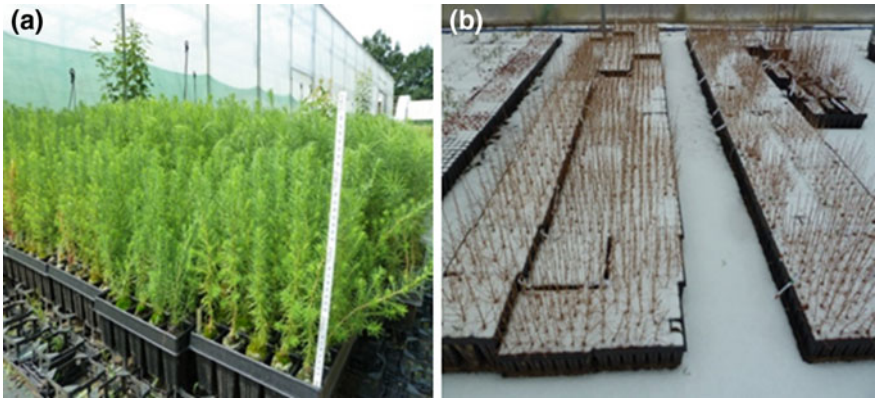

Fig. 5 Young plants of *Larix* × *eurolepis* derived from somatic embryogenesis. **a** Potted plants in the nursery (Staatsbetrieb Sachsenforst). **b** Overwintering in the nursery

3. Cultivation and growth control of young plants: Once the plantlets have rooted through the whole Mini-Jiffy (Fig. 4c), they may be potted in a regular conifer potting mix and immediately transferred to the nursery. This requires an initial frost free period for at least 6–8 weeks. Growth is controllable by the size of the pots as well as the amount of fertilizer applied. Quickpot plates of the size QP35T (50 × 50 × 115 mm per pot, 35 pots per plate) are very suitable. They are sufficiently large to support the young plants for up to one year (Fig. 5a). Complement weekly with a 1–2% liquid fertilizer during the vegetation period. Young plants remain in the nursery until they have reached a size of 30–50 cm and have overwintered once (Fig. 5b). At this point the plants are ready to be lined out in the forest plantation site (Fig. 6a, b).

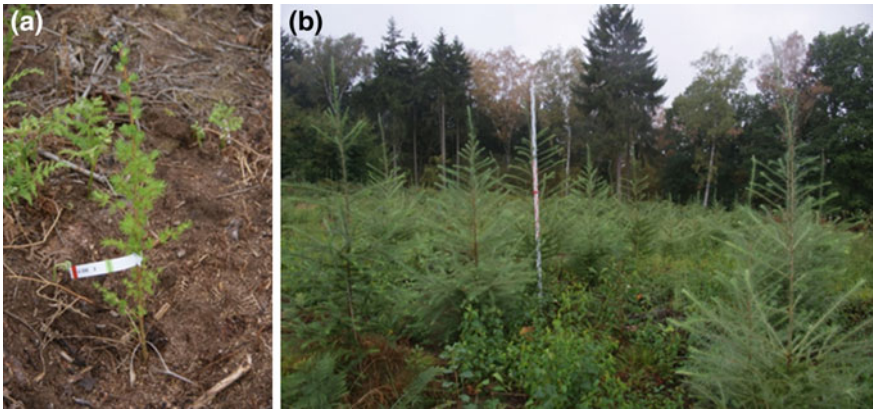

Fig. 6 *Larix* × *eurolepis* derived from somatic embryogenesis in the field. **a** At the time of planting, Height appr. 40 cm. **b** One year after planting. Height appr. 2 m

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Aleppo pine *Pinus halepensis* Mill.

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1 Introduction

Aleppo pine (*Pinus halepensis* Mill.) is a medium size tree (20–40 m) that can display a shrubby phenotype when growing on harsh conditions (Eckenwalder 2009). Female cones present a biannual maturation with a limited development in the first summer and autumn and a second phase of development, after fertilization, on the second summer (Puértolas Simón et al. 2012).

This species is native from the Mediterranean area and is widespread from Spain to Algeria (Botella et al. 2010) where, due to its very specific temperature and precipitation requirements, its distribution is generally confined to small areas (Klein et al. 2011). In Southern Europe occurs in several countries such as Spain, Portugal, France, Italy and Greece and in Northern Africa in Morocco, Algeria and Tunisia.

Like most of the Mediterranean conifers, has a mandatory regeneration by seed, since there is no regrowth from the trunk and branches. The seeds are resistant to high temperatures, even requiring this stimulus to germinate, usually after forest fires, and darkness also accelerates germination rates (Calvo et al. 2013). Its regeneration capacity after fires has been extensively exploited throughout most of the Mediterranean area (Osem et al. 2013).

This species has eco-physiological characteristics that assure tolerance to dry conditions such as a fast-growing root system, a water-saving strategy and resistance to water-drought interaction that allow it to live in a wide variety of soils,

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where others arboreal species have difficulties to establish (Puértolas Simón et al. 2012). As a pioneer species that facilitates the long-term colonization and development of late-successional species (Gil and Aránzazu 1993; Montero and Alcanda 1993) it is plausible that it might be used in large afforestation in the future (Oliveras et al. 2003; Maestre and Cortina 2004).

Traditionally, seed orchards provide genetically improved seeds, but, as described by Park et al. (1998) breeding strategies combined with in vitro vegetative propagation have shown advantages, including additional genetic gain and the possibility of introducing clones to meet market goals at a higher speed. In this sense, propagation via somatic embryogenesis (SE) in conifers led to a significant interest in establishing efficient regeneration protocols for this group of plants with high economic importance throughout the world (Bonga et al. 2010). This system offers the capability to produce large numbers of somatic embryo derived plantlets (Montalbán et al. 2010). Recently, many studies have been made in order to improve the different stages of SE systems in *Pinus* spp. (García-Mendiguren et al. 2016; Montalbán et al. 2010, 2011).

The only studies conducted in SE of Aleppo pine was carried out by Montalbán et al. (2013); the effect of culture medium (mineral salts, nitrogen source and plant growth regulators), collection date and seed family on embryogenic tissue initiation and proliferation were analysed. Recently, Pereira et al. (2016), analysed the effect of physical and chemical conditions, such as different water availability and temperatures, at initiation stage of *P. halepensis* somatic embryogenesis on the final success of the process.

2 Protocol of Somatic Embryogenesis in Aleppo PINE

The following protocol is the procedure described in Montalbán et al. (2013).

2.1 Culture Medium

The basal medium used for Aleppo pine tissue culture is DCR medium (Gupta and Durzan 1985) with some modifications and additives as specified in Table 1. This basal medium is used throughout the protocol, except for the germination stage, in which half-strength macronutrients LP (Quoirin and Lepoivre 1977, modified by Aitken-Christie et al. 1988) medium with some additives is used (Table 1).

Adjust the medium to pH 5.7 and autoclave it at 121 °C for 20 min. Note that the EDM amino acid mixture (Smith 1996; Table 1), once its pH is adjusted to 5.7 and filter-sterilized, must be added to the cooled autoclaved basal medium, since high temperatures may affect structure of the amino acids.

Add different additives and concentration accordingly to each stage throughout the process (Table 1).

Table 1 Specific basal medium and additives for the different media used during different stages of *P. halepensis* somatic embryogenesis

	Stage	Hormones (μM)	Agar (g L^{-1})	EDM amino acid mixture	Activated charcoal (g L^{-1})	Basal medium
1.	Initiation medium	2,4-D (9.0) kinetin (2.7)	Gelrite® 3	Yes	–	DCR
2.	Proliferation medium	2,4-D (9.0) kinetin (2.7)	Gelrite® 4.5	Yes	–	DCR
3.	Growth regulator-free DCR medium	–	–	Yes	–	DCR
4.	Maturation medium	ABA(75)	Gelrite® 9	Yes	–	DCR
5.	Germination medium	–	Difco® agar 9.5	No	2	Half-strength macronutrients LP

2,4-D—2,4-dichlorophenoxyacetic acid, ABA—abscisic acid

2.2 Explant Preparation

1. Select healthy one-year-old green female cones from mother plants; spray them with 70% (v/v) ethanol and cut them in four pieces to remove all immature seeds. Cones collected during the first week of August, corresponding to early cleavage polyembryony and the first "bullet" stages with a dominant embryo (Hargreaves et al. 2009) must be selected to perform the assays.
2. Surface sterilize the immature seeds in H_2O_2 10% (v/v) with two drops of Tween 20® for 10–15 min, and then rinse three times with sterile distilled H_2O. Note that from this step forward a careful manipulation under sterile conditions in the laminar flow unit is fundamental.
3. Using sterilized tweezers and a scalpel, on autoclaved paper, remove the whole megagametophytes containing immature embryos from the sterilized seeds. A reduced number of seeds manipulated for paper sheet, prevent seeds from drying and continuous sterilization of materials during manipulation reduces the risk of contamination.

2.3 Embryogenic Masses Initiation and Proliferation

1. Culture the megagametophytes on Petri dishes with 20 mL of the initiation medium specified in Sect. 2.1. Whole megagametophytes containing immature embryos must be horizontally placed onto the medium. Because of potential latent internal contamination, a Petri dish must have a maximum of eight megagametophytes, which one carefully dispersed following the direction of the laminar flow, avoiding contact with others.
2. Samples must be stored in a growth chamber at 23 °C in darkness. Note that about 3–5 weeks of culture embryogenic masses start to appear at one pole of the megagametophyte (Fig. 1a).
3. After 5–10 weeks on the initiation media, separate the proliferating embryogenic masses with an approximate diameter of 16 mm (Fig. 1b) from the megagametophytes.
4. Transfer the proliferating embryogenic masses to a Petri dish with 20 mL of proliferation medium specified in Sect. 2.1.
5. Samples must be subcultured every two weeks, and stored in a chamber at the same temperature (23 °C), in the dark. In order to maximize proliferation potential, subcultures must be made with peripheral tissue that tends to be more active (Fig. 1c).

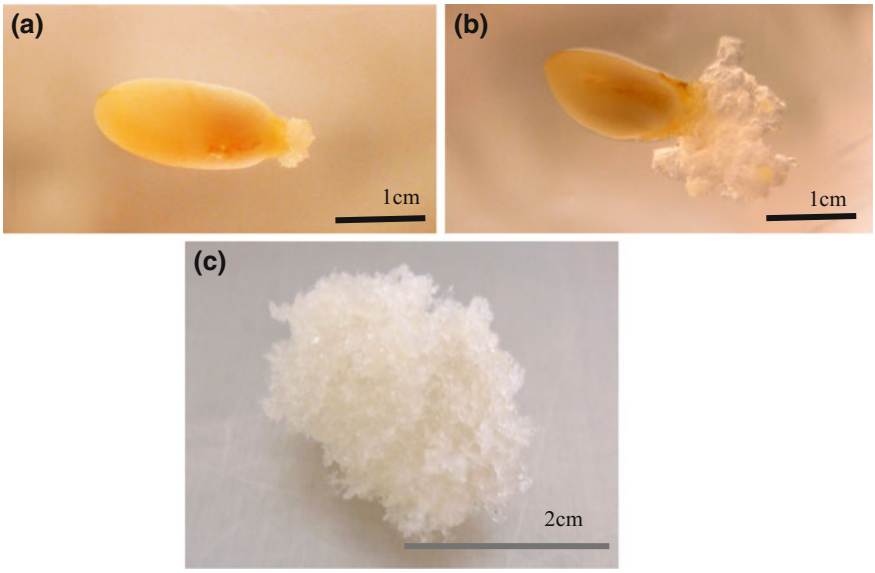

Fig. 1 *Pinus halepensis* embryogenic masses development. **a** Embryogenic tissue appearing at one pole of the megagametophyte; **b** embryogenic mass which must be further transferred to the proliferation medium; **c** high proliferative embryogenic mass isolated at proliferation medium

2.4 Somatic Embryo Maturation

1. Suspend embryogenic tissue in 20 mL of liquid growth regulator-free DCR medium and homogenize the suspension manually shaking it for a few seconds.
2. With a sterile 10 mL pipette transfer 5 mL aliquots containing 80 mg fresh weight and pour it onto a filter paper disc (Whatman no. 2) upon a Büchner funnel connected to a side-arm flask by a black rubber adaptor. Spread the tissue all over the paper disc in order to avoid overgrow and assure a better distribution of nutrients.
3. Apply a vacuum pulse for 10 s and transfer the filter paper with the attached embryogenic tissue to a Petri dish (90 × 20 mm) containing 40 mL of maturation medium described in Sect. 2.1.
4. Cultures should be putted in the growth chamber at 23 °C in the dark, for about four months to achieve somatic embryo development.

2.5 Germination and Acclimatization

After 15 weeks, collect mature somatic embryos, i.e., white to yellowish non-germinating somatic embryos with a distinct hypocotyl region and at least three

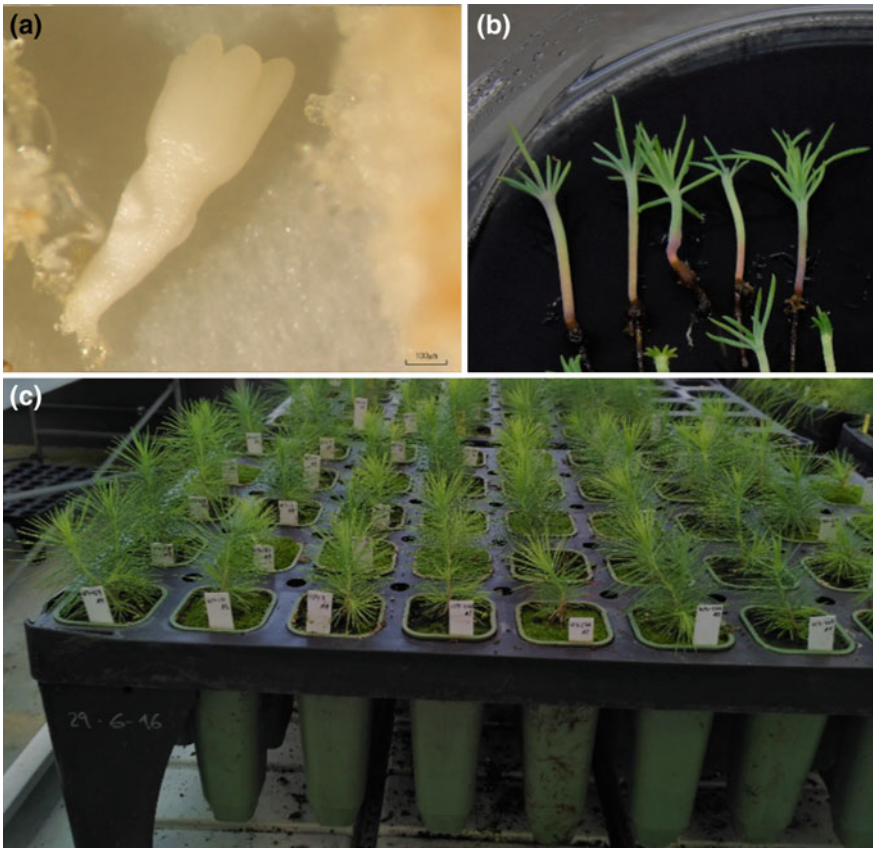

Fig. 2 *Pinus halepensis* somatic embryos maturation and germination; **a** Aleppo pine non-germinating mature somatic embryo; **b** somatic embryos on germination medium with embryonal root caps pointing downwards; **c** plantets acclimatization

cotyledons (Fig. 2a), and transfer those to a Petri dish containing 20 mL of germination medium described in Sect. 2.1.

Culture somatic embryos on Petri dishes with embryonal root caps pointing downwards (Fig. 2b) and tilt the Petri dishes vertically at an angle of approximately 45°–60°. The cultures are maintained at 21–24 °C under a 16-h photoperiod at 40 μmol m^{-2} s^{-1} for 1 to 2 weeks, and then at 120 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes.

After 6–8 weeks on germination medium, subculture the plantlets once onto fresh germination medium. After another 6–8 weeks on germination medium, transfer the somatic plants to trays with a sterile potting mix. As potting mix, use a peat:perlite, ratio 7:3.

Acclimatize the plantlets in a greenhouse under controlled conditions decreasing humidity progressively (Fig. 2c).

3 Research Prospects

In light of expected climate changes, with global drying and warming, that will negatively affect tree growth and vigour, there is an immediate need to improve tree productivity and the efficiency of forest systems. Due to its eco-physiological characteristics *P. halepensis* must have a major importance in future afforestation and a commercially efficient protocol of micropropagation in this species should be improved. Contrary to other species, studies of SE in this species are at a very preliminary stage, leaning so far at the initials stages of the process. Since all different steps of SE influence the final success, optimization of protocols concerning physical and chemical conditions throughout all the different stages are being topics of several studies in order to improve an efficient multi-step protocol.

Lastly, an effective cryoconservation protocol for *P. halepensis* embryogenic tissue could enable the preservation of its potentiality, and, as consequence, a continuous production of selected elite clones.

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Maritime Pine Pinus Pinaster Aiton

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1 Introduction

Maritime pine (*Pinus pinaster* Aiton) is the most abundant conifer in the Mediterranean basin occurring naturally from southwestern Europe (France, Portugal, Spain, and Italy) to northwestern Africa (Algeria, Tunisia, and Morocco). The ecological variability of maritime pine habitats and the geographical isolation among its populations give raise to several geographic races, which present differences in their genetic characteristics, and morphological and adaptive traits (Wahid et al. 2006; de Miguel et al. 2014).

Besides its ecological value, the species is of significant economic importance due to its uses for pulp and paper production, wood products and resin extraction (Alía and Martín 2003). Most recently, pycnogenol, a product that contains antioxidants, from maritime pine bark extracts is being widely studied for its pharmacological properties (Ying-Ya et al. 2015). Because of this, the species has also been introduced to Australia, New Zealand, Argentina, and Chile.

Breeding programs were initiated in the middle sixties of the nineteen-century in France and Portugal and latter on in Spain (see references in Humánez et al. 2011). Currently, maritime pine is considered to be a model conifer species for study of the adaption responses to drought stress from a genomics approach. Thus, its large

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genome, estimated on 30,000 Mb, is being sequenced in the frame of the European project ProCoGen (www.procogen.eu) and other collaborative initiatives (Mackay et al. 2012) using haploid lines from maritime pine megagametophytes (Arrillaga et al. 2014; Cabezas et al. 2016). In this context, the availability of protocols that allow not only mass vegetative propagation of selected families or genotypes, but also facilitate the functional analyses needed to verify and further to study the effects of candidate genes are necessary.

In vitro propagation of the species has been reported through axillary bud breaking (de Diego et al. 2008; Humánez et al. 2011), adventitious bud differentiation (David et al. 1982; Calixto and Pais 1997; Tereso et al. 2006; Alvarez et al. 2009; Humánez et al. 2011) and somatic embryogenesis (Miguel et al. 2004; Lelu-Walter et al. 2006; Klimaszewska et al. 2007; Humánez et al. 2012); among these, somatic embryogenesis (SE) is the primary enabling technology required to achieve breeding objectives in conifers.

Maritime pine SE has been developed from isolated immature embryos and/or megagametophytes containing the immature zygotic embryo from French (Lelu-Walter et al. 2006), Portuguese (Miguel et al. 2004), and Spanish (Humánez et al. 2012) half-sib families. Although these protocols allow the generation of plants from most of the half-sib family tested, differences among the embryogenic capability of several maritime pine provenances has also been described (Morcillo et al. 2012). These authors reported higher embryogenic potential in maritime pine from the Atlantic origin. SE technology has also been used to study candidate genes for adaptative traits using cDNA sequences from transcriptome data (Trontin et al. 2012; Canales et al. 2014). In spite of the efforts addressed to clone adult maritime pine genotypes through SE, this goal remains still a challenge (Humánez et al. 2012; Lelu-Walter et al. 2016).

One of the main factors affecting somatic embryogenesis induction in pines is the developmental stage of the zygotic embryo in the initial explants. Thus, optimal embryogenic response in maritime pine occurs when the dominant zygotic embryo had begun to develop (Humánez et al. 2012). Embryogenic lines from maritime pine are easily established by culturing megagametophytes on modified Litvay (Litvay et al. 1985) or DCR (Gupta and Durzan 1985) media containing 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyladenine (BA). No differences between media in terms of the number of SE lines established after 4 months in culture have been observed (Humánez et al. 2012). In contrast, the yield and quality of somatic embryos obtained after maturation phase strongly depends on the line.

Here we describe an improved protocol based on those previously reported (for references see Humánez et al. 2012) to generate maritime pine plants through somatic embryogenesis from immature megagametophytes. Procedures including explant preparation, somatic embryo induction, proliferation, cryopreservation, maturation, germination, plant formation, and acclimatization are described.

2 Protocol of Somatic Embryogenesis in Maritime Pine

The regeneration procedure includes several steps: (1) induction of somatic embryogenesis from megagametophytes, (2) proliferation of embryonal masses (EMs) (maintenance of cultures), (3) cryopreservation and cell recovery, (4) somatic embryo maturation, (5) somatic embryo germination and root development, and (6) transfer to ex vitro conditions and acclimatization of the regenerated somatic seedlings.

2.1 Material

1. Plant materials as source of explants: maritime pine female cones, from controlled crosses or open pollinated trees, collected from middle July to early August, when seeds contain megagametophytes in which the dominant zygotic embryo had begun to develop (Fig. 1a–d).
2. Laminar-flow hood with ultraviolet light, glass bead sterilizer, forceps and scalpels.
3. Precision balance, autoclave, stereoscopy microscope and shaking incubators.
4. Ethanol (96%) and sterile distilled water.
5. Cryogenic container, liquid nitrogen and chemicals for cryopreservation: dimethyl sulfoxide (DMSO), polyethylene glycol (PEG-4000).

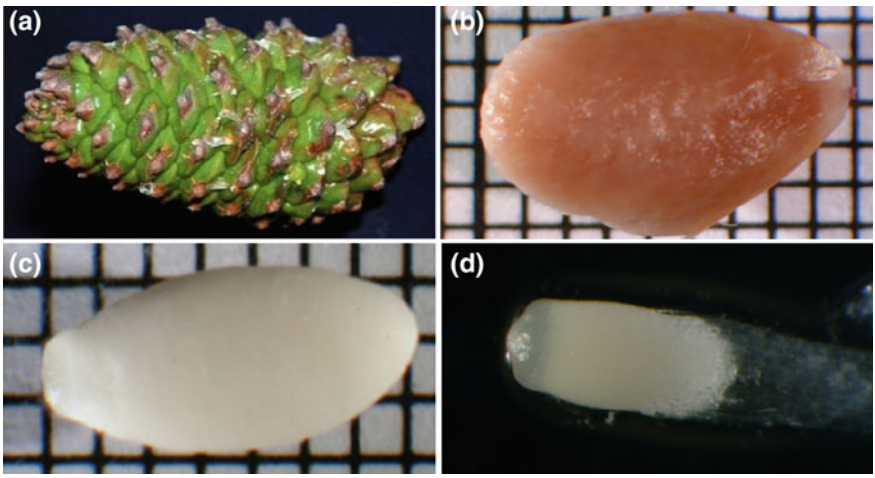

Fig. 1 Plant material for induction of maritime pine somatic embryogenesis. **a** Immature cone collected in middle-end July; **b** isolated seed; **c** isolated megagametophyte comprising the zygotic embryo at the precotiledonary stage (**d**)

6. Sterile glassware: sterile 1000–5000 mL beakers, 1000–5000 mL Erlenmeyer flasks, Büchner funnels and Kitasato flasks.
7. Culture vessels of different types: sterile Petri dishes (100 × 20 mm or 15 × 90 mm) and 500 mL glass jars.
8. Sterile 50 mL centrifuge tubes, pipettes (1–25 mL), air-displacement piston pipettes (2–1000 µL), single use filters (22 µm). Whatman qualitative filter paper Grade 2 (70 Ø mm) circles, and sterile filter paper (20 × 20 cm).
9. Parafilm®, 3 M™ Micropore™ Surgical Tape.
10. Mineral salts, plant growth regulators (PGR), organics and other substances for media preparation (see Tables 1 and 2): 6-benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), abscisic acid (ABA, Sigma, St Louis, Missouri, USA); Gelrite (G1910, Sigma, Aldrich), BD Bacto™ Dehydrated agar (Fisher Scientific), sucrose, myo-inositol, casein hydrolysate, L-glutamine, activated charcoal (C9157, Sigma Aldrich).
11. Tissue culture growth chambers.
12. Forest containers, kekkila substrate, perlite (substrate:perlite, 7:3).

Basal media composition is listed in Table 1. Required modifications for different culture steps are listed in Table 2. The pH is adjusted to 5.7–5.8 with KOH or HCl prior to autoclaving at 121 °C for 20 min, 10^5 Pa. Glutamine and ABA are filter sterilized and added to media after autoclaving. Pour 25 or 30 mL into 90 × 15 or 90 × 20 mm Petri dishes, respectively, and 50 ml medium into sterile glass jars.

Table 1 Maritime pine basal culture media

Chemicals	Quantity in mg/L		Chemicals	Quantity in mg/L	
	mLV	DCR		mLV	DCR
Macronutrients			Micronutrients		
$Ca(NO_3)_2.4H_2O$	–	556	$ZnSO_4.7H_2O$	43	8.6
KNO_3	950	340	$CoCl_2.6H2O$	0.125	0.025
$CaCl_2.2H_2O$	8.3	85	$CuSO_4.5H_2O$	0.50	0.25
NH_4NO_3	825	400	$FeSO_4.7H_2O$	27.8	27.8
$MgSO_4.7H_2O$	451.69	370	Na_2EDTA	37.3	37.3
KH_2PO_4	170	–	H_3BO_3	31	6.2
			KI	4.15	0.83
			$MnSO_4{\cdot}4H_2O$	21	22.3
			$NiCl_2{\cdot}6H_2O$	–	0.025
Organic supplements			$Na_2MoO_4{\cdot}2H_2O$	1.25	0.25
Myo-inositol	100	200			
Nicotinic acid	0.5	0.5			
Pyridoxin HCl	0.1	0.5			
Thiamine HCl	0.1	1			
Glycine	–	2			

The pH was adjusted to 5.7–5.8 with KOH or HCl prior to autoclaving at 121 °C for 20 min

Table 2 Formulations of Maritime Pine media

Chemicals	M1	M2	M3	M4	M5	M6
	Initiation & maintenance	Pre-maturation	Maturation	Germination	Post-germination	Plantlet development
Basal medium	mLV	mLV	mLV	mLV	DCR (1/2)	DCR (macronutrients/2)
PGR (μM)						
2,4-D	8.8	–	–	–	–	–
BA	4.4	–	–	–	–	–
ABA	–	–	80.0	–	–	–
Other (g L^{-1})						
Sucrose	20.0	60.0	60.0	30.0	20.0	20.0
Casein hydrolysate	1.0	1.0	1.0	1.0	1.0	–
L-Glutamine	0.625	0.625	0.625	0.625	–	–
Activated charcoal	–	–	–	–	10.0	–
Agar[(1)]	4.0[(a)]	9.0[(a)]	9.0[(a)]	4.0[(a)]	4.0[(a)]	7.5[(b)]

[(1)]Agar, not used for liquid media. (a) Gelrite, Gelzan™, (b) BD Bacto™
The pH of all media is adjusted to 5.7–5.8

2.2 Embryogenic Culture Initiation

1. Collect female cones in middle July to early August when the dominant zygotic embryo had begun to develop (Fig. 1a).
2. Sterilize the cones with ethanol 96% for 20 min.
3. Discard the ethanol and let the cones dry for 15 min on sterile filter paper in the laminar-flow-hood.
4. Using sterile scalpels and forceps remove the seeds from the cones (Fig. 1b).
5. Transfer the seeds into a Petri dish.
6. Remove the seed coat with sterile scalpel and forceps and aseptically isolate the megagametophyte under a dissecting microscope (Fig. 1c, d).
7. Place the isolated megagametophytes on the surface of 25 mL of semisolid embryogenic induction medium (M1) in 15 mm × 90 mm Petri dishes.
8. Incubate the megagametophytes in darkness at 23 °C.

2.3 Maintenance and Proliferation of Embryogenic Cultures

1. Callus is first visualized as an extrusion near the micropyle of the cultured megagametophytes within 4 weeks on M1 (Fig. 2a).
2. Select extruded embryogenic callus and transfer to M1 medium for further proliferation. Subculture to fresh medium of the same composition every two weeks.
3. After at least three subcultures, white mucilaginous, spiky, embryogenic callus containing embryogenic suspensor masses (ESMs) is observed and the embryogenic line is established (Fig. 2b).

Maintain the established ESMs cultures by two weeks subcultures to fresh M1 medium. Use this material for proliferation, cryopreservation and maturation steps.

2.4 Cryopreservation and Recovery of the Embryogenic Lines

ESMs will remain embryogenic for about six months. Aged cultures will not produce mature somatic embryos, and then cryopreservation is an alternative to maintain embryogenic lines for further experiments. The protocol is adapted from (Marum et al. 2004).

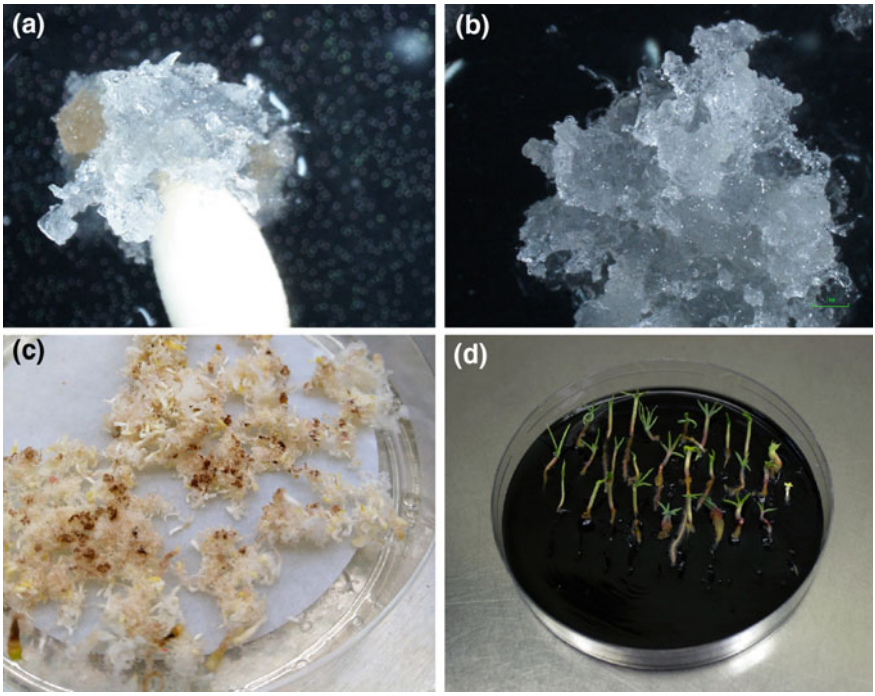

Fig. 2 Embryogenesis process on maritime pine. **a** Extrussion of embryogenic cells from megagametophyte mycropile; **b** establishment of maritime pine embryogenic line; **c** somatic embryo maturation on ABA-containing medium; **d** germination of SE on activated charcoal containing (M5) medium

1. Transfer 3 g of actively growing embryogenic cell masses (10 days after the last subculture) to 10.8 mL of liquid M1 medium without plant growth regulators, in 125 mL culture flasks.
2. Place the flasks on a shaker for disaggregation of the masses, at 23 °C in dark for 24 h at 90 rpm.
3. Add 1.2 mL of filter-sterilized maltose (2 M) dropwise over a period of 30 min until a 0.2 M is reached. Return flasks to the shakers and maintain them in the dark.
4. On the second day, remove carefully 1.2 mL of medium from the flasks (avoiding the uptake of cells) and add another 1.2 mL of the maltose stock solution as previously described to reach a final concentration of 0.4 M. Return flasks to the shakers and maintain them in the dark.
5. On the third day, transfer the flasks containing the embryogenic suspensions to ice and, after 15 min, add 12.6 mL of PSD solution (M1 proliferation medium without PGR supplemented with 10% Sucrose, 10% PEG-4000 and 10% DMSO) dropwise over a period of 30 min.

6. Dispense 1.8 mL aliquots into 2 mL cryovials and place them in a Nalgene® Cryo 1 °C "Mr. Frosty" Freezing Container (filled with isopropyl alcohol and previously incubate at −80 °C for 24 h). Introduce the freezing container in a −80 freeze.
7. After 24 h, plug cryovials into liquid nitrogen (−196 °C) for their storage.

Cell recovery

8. Remove cryovials from liquid nitrogen and shortly place them into a Thermoblock at 40 °C until thawing, and then transfer cryovials to ice.
9. Surface sterilize cryovials with 96º ethanol and allow them to dry in a laminar flow.
10. Plate the content of each cryovial on autoclaved filter paper discs (70 mm Ø, Whatman Grade 2) placed on the top of various layers of sterile filter paper.
11. Wash the cells with 5 mL of MI medium without PGR.
12. Transfer the filter paper disc onto MI medium. After 24 h, transfer the filter paper disc to fresh medium.
13. Transfer filters with recovered callus to fresh medium every 2 weeks until tissue recovery. Proliferate tissue as in 2.3.

2.5 Embryo Maturation

1. Weight 110–170 mg of spiky ESMs and transfer them into 50 mL centrifuge tubes containing 4 mL of liquid M1 without PGR.
2. Place the tubes on a shaker or shake them manually until cell suspension is homogenous.
3. Collect the ESMs cells on a filter paper disc (Whatman No. 2, 7 mm Ø) in a Büchner funnel coupled to a Kitasato flask and let flow-trough to drain. When appropriate, low-pressure vacuum pulse can be applied to drain the liquid.
4. Transfer the filter paper containing the disaggregated ESMs to prematuration M2 medium.
5. After 10 days, transfer the filter paper to ABA-containing M3 medium. Seal the plates with film and incubate them at 23 °C in dark.

2.6 Embryo Germination

1. After 12 weeks on M3 medium, collect early cotiledonary and mature embryos under dissecting microscope and transfer them to plates with germination M4 medium (Fig. 2c). Seal the plates with 3 M™ Micropore™ Surgical Tape and place them vertically at an angle of 60 °C at 23 °C in the dark.

Fig. 3 Maritime plants developed on post-germiantion medium (**a**) and after two months after transferring to the greenhouse (**b**)

2. After 10 to 15 days, transfer germinated embryos with elongated hypocotyls to plates with M5 medium and maintain them in the previously described conditions but under dim light until a powerful root develops (approximately 1 month) (Fig. 2d).
3. Transfer plantlets to glass jars containing 50 mL of M6 medium. After 2 months, select morphologically normal plantlets with both shoots and roots for acclimatization (Fig. 3a).

2.7 Acclimatization and Transfer to Greenhouse

1. Transfer selected plantlets to square plastic containers (6 × 9 pots) with riblike structures containing a mixture of kekkila substrate: perlite (7:3 v/v).
2. Cover the square pot trays with a plastic bag to reach high humidity conditions (RH 90%) and maintain pots in a growth chamber at 25 °C and a 16 h photoperiod provided by mixed Sylvania Gro-Lux® and Philips cool-white fluorescent tubes (120–180 μmol m^{-2} s^{-1}) at 23 °C and 50% relative humidity. After one week, gradually expose the plants to growth chamber conditions by removing the covers for 2 additional hours every day. On the fifth day, perforate the plastic bags. After two more days (2 weeks after transfer to the growth chamber), remove the plastic bags and maintain plantlets for one additional week in the growth chamber.
3. Transfer the acclimatized plantlets to the greenhouse. To avoid an excessive dehydration, cover the plants with perforated plastic bags for the first week until plants re-start growth. With this method more than 98% of the germinated embryos survive to the acclimatization phase (Fig. 3b).

3 Research Prospects

Pinus pinaster Aiton is one of the most commercial and economically valuable tree species in the Mediterrean Basin. Due to its capacity to live in a wide range of ecological conditions it has been traditionally used in breeding and reforestation programs but is also one of the most advanced models for conifer research.

Currently, maritime pine forest is threatened by extreme drought and pathogen attacks as *Fusarium* spp and *Tomicus* spp (Vivas et al. 2013; Grégoire and Evans 2004). Two approaches have been suggested for increasing their resilience: breeding for resistance and to stimulate induced defense/adaptation responses. SE biotechnology can shorten traditional breeding methods by cloning resistant individuals that may maintain the resistance in the clonal offspring. In addition, genes of resistance can be introduced by direct genetic modification in somatic embryos producing transgenic resistant plants.

In spite of efforts conducting to clone select adult trees, to date however only callus of embryogenic appearance have been produced, but these tissue failed to proliferate (Humánez et al. 2012). New strategies such as the use of novel plant growth regulators as sulphokines and/or adult material established in vitro should be approached (Umehara et al. 2005). Also the increasing knowledge on the genes that regulate the embryogenic process in conifers (Morel et al. 2014) will help to early determine the embryogenic identity of the induced callus. Successfully protocols to clone adult trees may also take advantage from the molecular markers (such as endogenous hormone levels or gene expression) that could be used to verify the quality of the embryogenic suspensor masses (ESMs) prior to their maturation. Preliminary experiments associate high levels of endogenous ABA content, in the proliferating ESMs with lines that do not respond to further ABA treatments during maturation step (Arrillaga, personal communication). Also correlation into gene expression of genes and maturation capability is under study (Lelu-Walter et al. 2016).

During the last years forest environmental-induced epigenetic changes have been shown to mediate phenotypic plasticity by regulation of specific gene expression (Saez-Laguna et al. 2014). Moreover, acquisition of this epigenetic memory has been linked with the process of seed formation and zygotic embryo development. Overall, this is called transgenerational epigenetic stress memory (Holeski et al. 2012) and could, therefore be mimetised by means of somatic embryogenesis. Then, is likely that clonal plants regenerated from embryogenic lines that underwent different environmental stresses may display some memory of response to the stress. This hypothesis is being proving in maritime pine (Arrillaga et al. 2016).

Genome sequence of maritime pine will allow the identification of DNA sequences of candidate genes that along with the propagation and gene transfer protocols for the species may speed up its genetic improvement (https://procogen.wordpress.com/). In addition, the sequence DNA data obtained from this project will also provide a unique and exciting opportunity to test newly developed theory

for the study of adaptive evolution and genetic diversity both within and between species using the family Pinaceae as a model plant taxa.

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Holm Oak *Quercus ilex* L.

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1 Introduction

The holm oak is an evergreen tree species of the Fagaceae family. This species is one of the most important components of the Mediterranean forests.

Its natural distribution range extends over several circum-Mediterranean European and African countries, and it is extending northwards as consequence of climate warming (Delzon et al. 2013). The largest populations of this species are in the Iberian Peninsula. Conjointly with other oak species, mainly *Quercus suber*, the holm oak constitutes the tree cover of an agro-silvo-pastoral system called "dehesa" in Spain and "montado" in Portugal. These savanna-like open woodlands spread over about four million hectares (Sá-Sousa 2014) and are protected by the Council Directive 92/43/EEC amended by the Directive 2013/17/EU of the European Union.

In addition to its ecological relevance, the holm oak is a species of high economical interest for the development of the rural populations of its distribution area. Its wood has been used in the past in furniture and for making charcoal, but the main current product is the acorn crop. Acorns are used for feeding a special livestock breed, the Iberian black pig, which is the basis of a highly appreciated

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gastronomical industry (Rey and López-Bote 2014). Furthermore several bioactive compounds have been identified in acorns of oak species that envisage new potential industrial applications in nutrition, pharmacology and cosmetics (Vinha et al. 2016). The holm oak is also been used to establish plantations with plants mycorrhized with edible fungus, particularly the highly demanded black truffle (Reyna and Garcia-Barreda 2014).

The holm oak is highly threatened by several factors such as forest fires and poor natural regeneration. However the most important hazard is a decay syndrome called "la seca", mainly caused by root infection by *Phytophthora cinnamomi*, which is causing a high mortality in the last years (de Sampaio e Paiva Camilo-Alves et al. 2013). Therefore the establishment of plantations with genetically improved materials is desirable. Within the strategies of genetic improvement programs for oaks, vegetative propagation plays a main role (Savill and Kanowski 1993). Vegetative propagation allows the benefits of breeding to be rapidly and flexibly transferred to improved plant material that can be deployed in intensively managed plantations (Lelu-Walter et al. 2013). Somatic embryogenesis (SE) is considered the enabling technology for performing high-value multi-varietal forestry, i.e., the use of genetically tested clones in plantation forestry, balancing genetic gain and diversity (Park 2014).

The holm oak is considered as a recalcitrant species to vegetative propagation by root cuttings, which is highly conditioned by the donor plant age (L'Helgoual'ch and Espagnac 1987). Plant regeneration by organogenesis from in vitro holm oak germinated seedlings (Liñán et al. 2011) and by SE from immature zygotic embryos (Mauri and Manzanera 2003) was reported. However, in forest species is of paramount importance to be able to clone plants from tissues of adult donor trees. This is because correlations between traits at juvenile and adult stages are usually rather poor, precluding a reliable early selection. Species of the *Quercus* genus are particularly suited for the induction of SE in tissues from adult trees (Corredoira et al. 2014).

Since protocols of SE in tissues of young origin have been described in the former book of this series (Mauri and Manzanera 2005) the present chapter focuses on SE protocols in tissues from adult trees. Unlike other oak species such as *Quercus suber* and *Quercus robur* in which SE was induced in leaves from epicormic shoots flushed from pieces of branches collected from adult trees (Corredoira et al. 2014), the holm oak was reluctant to SE using these explants (Blasco et al. 2013). Therefore, alternate explants were tested. The following protocols are based on research articles reporting SE induction in male catkins (Blasco et al. 2013), developing ovules (Barra-Jiménez et al. 2014) and apexes and expanding leaves from in vitro cultured shoots (Martínez et al. 2017). Protocols for the cryopreservation of embryogenic lines are also described (Barra-Jiménez et al. 2015).

2 Protocol of Somatic Embryogenesis in Holm Oak

The regeneration procedure includes three steps: (1) induction of somatic embryogenesis from different explants (culture initiation), (2) proliferation of embryogenic lines (maintenance of cultures), and (3) somatic embryo germination and plantlet conversion. The last step is the transfer to ex vitro conditions and the acclimatization of the regenerated somatic seedlings.

2.1 Materials

1. Plant materials as source of explants: developing catkins, immature acorns or axillary shoot cultures from epicormics shoots forced from branch segments from the crown of adult trees.
2. Ultrapure water Milli-Q, ethanol (96 and 99 °C), 1000–5000 mL Erlenmeyer flasks. Commercial bleach (3.5% active chlorine). Tween® 20 (Sigma-Aldrich, USA, ref. P2287).
3. Mineral salts, plant growth regulators (PGR), organics and other substances for media preparation (see Tables 1 and 2): 6-benzyladenine (BA), indole-acetic acid (IAA), indole-butyric acid (IBA), naphthalene-acetic acid (NAA), Agar (Sigma-Aldrich, USA, ref. A-1296), Vitro agar (Pronadisa, Spain), Plantagar S1000 (B&V, Italy), Plant Agar (Duchefa, The Netherlands, ref. P1001), sucrose, myo-inositol, silver thiosulphate (STS; sodium thiosulfate, silver nitrate), casein hydrolysate.
4. Cryogenic container, liquid nitrogen and chemicals for cryopreservation: dimethyl sulfoxide (DMSO), glycerol, ethylene glycol.
5. Pipettes (1–25 mL), air-displacement piston pipettes (2–1000 μL), single use filters (22 μm), cryovials.
6. Precision balance and autoclave.
7. Culture vessels of different types: 15 × 55 or 90 mm Petri dishes, 500 mL glass jars, 100-mL baby food jars (Sigma-Aldrich, ref. V8630), Sterivent High Containers (Duchefa, The Netherlands, ref. S1686). Parafilm®.
8. Laminar-flow cabinets with ultraviolet light. Glass bead sterilizer.
9. Stereoscopy microscopes, forceps, scalpels.
10. Rotary microtome, transmission light microscope, and chemicals for microscopy: formalin, glacial acetic acid, n-butanol, paraffin wax, safranin, fast green, periodic acid-Schiff (PAS) kit, naphtol blue-black.
11. Tissue culture growth chambers.
12. Forest containers. Peat, coconut fiber, perlite, slow-release fertilizer Osmocote™. Fungicides: Previcur® (Propamocarb 60.5%), Benoagrex® (Benomyl 50%), Captamur® (Captan 47.5%), Laincobre® (Mancozeb 17.5% + $Cu_2(OH)_3Cl$ 22%).

Table 1 Basal culture media used for plant regeneration of *Quercus ilex* by somatic embryogenesis

Chemicals	BM1[a]	BM2[b]	BM3[c]	BM4[d]	BM5[e]	BM6[f]
$CaCl_2$	72.50	332.02	332.02	151.00	–	–
$CaCl_2 \cdot 2H_2O$	–	–	–	–	150.00	150.00
$Ca(NO_3)_2 \cdot 4H_2O$	471.26	–	–	–	–	–
$Ca(NO_3)_2 \cdot 2H_2O$	–	–	–	–	–	–
KCl	–	–	–	–	300.00	300.00
KH_2PO_4	170.00	170.00	170.00	–	–	–
K_2SO_4	990.00	–	–	–	–	–
KNO_3	–	1900.00	1900.00	2500.00	1000.00	1000.00
$MgSO_4$	180.54	180.54	180.54	195.05	–	–
$MgSO_4 \cdot 7H_2O$	–	–	–	–	250.00	250.00
Na_2HPO_4	–	–	–	–	30.00	30.00
$NaH_2PO_4 \cdot H_2O$	–	–	–	–	90.00	90.00
NH_4NO_3	400.00	1650.00	1650.00	–	–	–
$(NH_4)_2SO_4$	–	–	–	–	200.00	200.00
$(NH_4)H_2PO_4$	–	–	–	300.00	–	–
FeNaEDTA	36.70	36.70	36.70	36.70	36.70	36.70
$CoCl \cdot 6H_2O$	–	0.025	0.025	0.025	0.025	0.25
$CuSO_4 \cdot 5H_2O$	0.25	0.025	0.025	0.025	0.025	0.25
H_3BO_3	6.20	6.20	6.20	6.20	6.20	3.00
KI	–	0.83	0.83	0.83	0.83	0.75
$MnSO_4 \cdot H_2O$	22.30	16.90	16.90	16.90	16.90	10.00
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25
$ZnSO_4 \cdot 7H_2O$	8.60	8.60	8.60	8.60	8.60	3.00
Glycine	2.00	2.00	2.00	2.00	2.00	–
Myo-inositol	100.00	100.00	100.00	100.00	100.00	10.00
Nicotinic acid	0.50	0.50	1.00	0.50	0.50	0.10
Pyridoxine HCl	0.50	0.50	1.00	0.50	0.50	0.10
Thiamine HCl	1.00	0.10	10.00	0.10	0.10	1.00
pH	5.7	5.7	5.7	5.7	5.7	5.7

All units are in mg/L

[a] BM1, based on the Woody Plant Medium (WPM, Lloyd and McCown 1980)

[b] BM2, based on the Murashige and Skoog's MS medium (1962)

[c] BM3, based on the Murashige and Skoog's MS medium (1962) with modified vitamins

[d] BM4, macronutrients of Schenk and Hildebrandt's SH medium (1972) with micronutrients and vitamins of MS

[e] BM5, macronutrients of the Gresshoff and Doy´s GD medium (1972) with micronutrients and vitamins of MS

[f] BM6, based on the Gresshoff and Doy´s GD medium (1972) modified

Table 2 Culture media used for the induction of somatic embryogenesis in shoot apexes and expanding leaves of holm oak

Media components	Axillary shoot cultures	Embryogenic induction medium (M1)	Expression medium (M2, M3)
Basal medium	BM1	BM3	BM3
Sucrose	30	30	30
CH	–	0.5	0.5
STS	20	–	–
Agar (Sigma-Aldrich)	8	–	–
Vitro Agar (Pronadisa)	–	6	6
BA	0.44/0.044[a]	2.22	0.44/0[c]
Zeatin	0/0.45[a]	–	–
IAA or NAA	–	22.83	0.57/0[c]
NAA		21.48	0.54/0[c]
pH	5.7[b]	5.7	5.7

All concentration units are in g/L, except those of PGR and STS that are in μM

[a] 6-weeks multiplication cycle: 0.44 μM BA for the first 2 weeks, 0.22 μM BA for the next 2 weeks, and 0.044 μM BA plus 0.45 μM zeatin for the last 2 weeks

[b] The pH was adjusted to 5.7 with 0.5 N NaOH or 1 N HCl prior to autoclaving at 115 °C for 20 min

[c] Expression Medium (M2) with 0.44 μM BA plus 0.57 μM IAA or 0.54 μM NAA; Expression Medium (M3) without plant growth regulators

2.2 Culture Media

Basal media used for plant regeneration of holm oak by somatic embryogenesis are described in Table 1.

The media pH is adjusted to 5.7 with 0.5 N NaOH or 1 N HCl prior to autoclaving at 115 °C for 20 min or 121 °C for 30 min. Substances as STS are filter sterilized and added to sterile media aseptically.

2.3 Induction of Somatic Embryogenesis in Male Catkins

1. Collect holm oak unopened male flowers (catkins), 1–2.5 cm in length, between April and May depending on the climatic area (Fig. 1a, b).
2. Store samples in closed bags at 4 °C and darkness one day maximum to avoid fungal infections.
3. Disinfect catkins, distributed in small well tied muslin sachets, by submerging them in 70% ethanol for 2 min, and then in 8% commercial bleach solution

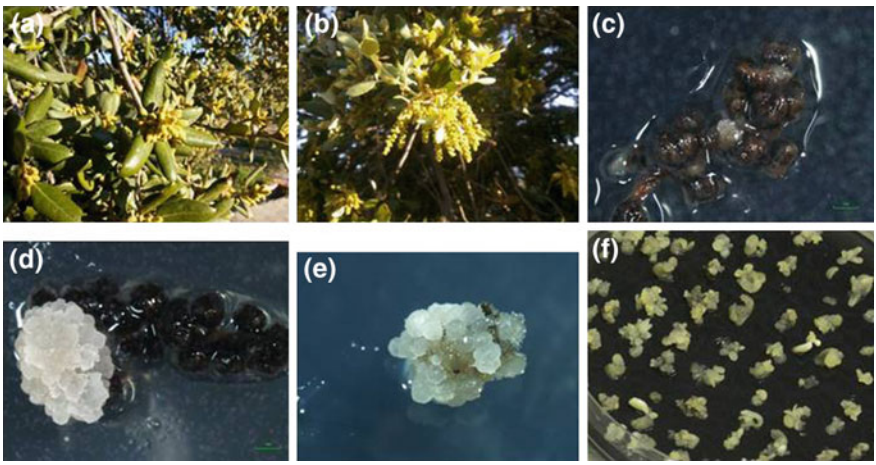

Fig. 1 Induction of somatic embryogenesis in male catkins of holm oak. **a** Immature male catkins with unopened flowers in April, **b** elongated catkins with unopened flowers in May, **c** callus arising from catkins after 1 month on induction medium, **d** pro-embryogenic masses (PEMs) generated on secondary induction medium, **e** detail of a nodular embryogenic clump obtained from catkins, **f** holm oak embryogenic line derived from catkins and established on medium supplemented with STS and active charcoal

containing two drops of Tween® 20 for 15 min applying strong agitation. Finally, rinse catkins sachets thrice with distilled water.

4. After sterilization place entire catkins into 90 mm diameter Petri dishes containing a 30 g/L sucrose solution with 7 g/L agar (Pronadisa) and maintain cultures in the dark at 25 ± 2 °C for 5 days. Then, discard necrotic or contaminated explants.
5. Transfer 10 catkins to each Petri dish containing 25 mL of a primary induction medium composed of basal medium BM2 with macronutrients at half strength, 10 μM BA and 50 μM NAA, 30 g/L sucrose and 8 g/L agar (Sigma-Aldrich).
6. Incubate the explants in darkness at 25 ± 2 °C for 30 days.
7. During this period, white callus will arise from the 62% of the genotypes tested (Fig. 1c). Eventually, individual somatic embryos could be differentiated.
8. Transfer explants to a secondary induction medium composed of BM4 basal medium with 30 g/L sucrose and 8 g/L agar (Sigma-Aldrich) without PGR. Incubate the dishes in a growth chamber at 25 °C and a 16-h photoperiod provided by mixed Sylvania Gro-Lux® and Philips cool-white fluorescent tubes (120–180 μmol $m^{-}2$ s^{-1}).
9. Subculture again onto fresh medium after 30 days and place the dishes under the same culture conditions until pro-embryogenic masses (PEMs) emerge (Fig. 1d, e).
10. Transfer PEMs or immature somatic embryos to proliferation medium composed of BM2 basal medium, 30 g/L sucrose, 0.5 g/L casein hydrolyzed,

20 μM STS, 4 g/L activated charcoal, 8 g/L agar (Sigma-Aldrich) and without PGR (Martínez et al. 2015).

11. Embryogenic lines will be established (Fig. 1f).

Alternatively PEMs or somatic embryos can be subcultured on BM2 medium with macronutrients at half strength and 60 g/L sorbitol to induce secondary embryogenesis. The protocol allows the capture of 12% of the genotypes tested.

2.4 *Induction of Somatic Embryogenesis in Teguments of Developing Ovules*

The fertilized ovule at an advanced stage of development is the starting explant.

1. Collect immature acorns, 8–9 mm diameter (Fig. 2a), with the developing fertilized ovule occupying the entire ovary cavity.
2. Disinfect the acorns (including cupules) by vigorous shake in 70% ethanol for 30 s, followed by immersion in a 15% solution of commercial bleach plus two drops of Tween® 20 for 10 min. Wash the acorns three times with sterile distilled water.
3. Strip the ovaries off the cupules and again immerse them in a 10% bleach solution plus two drops of Tween-20 for 5–10 min. Rinse the ovaries three times in sterile distilled water.
4. Dissect the ovaries and isolate the ovules enclosing zygotic embryos. The best responsive ovules are pale yellow in color, 3–4 mm width and 5–6 mm long. Take care to eliminate unfertilized ovules that usually are attached to the fertilized ovule (Fig. 2b).
5. Culture three explants in 55-mm diameter Petri dishes filled with 10 mL of BM4 basal medium, 30 g/L sucrose, 6 g/L agar (B&V) and lacking PGR. Seal the dishes with Parafilm®.
6. Place the dishes in a growth chamber at 25 °C in darkness.

Fig. 2 Induction of somatic embryogenesis in teguments of holm oak developing ovules. **a** Immature acorns with partially detached cupules to show the enlarging ovaries, **b** developing ovule excised from an ovary; note the remains of an unfertilized ovule, **c** formation of embryogenic masses arising from the teguments; bar = 3 mm

7. After 3–6 days in culture the zygotic embryos enlarge and broke the ovules. Remove the zygotic embryos and maintain in culture only the integuments.
8. After 30 days from the start, subculture the explants onto the same fresh medium and place the dishes in a growth chamber at 25 °C and a 16-h photoperiod provided by mixed Sylvania Gro-Lux® and Philips cool-white fluorescent tubes (120–180 μmol m^{-2} s^{-1}).
9. Subculture again onto fresh medium after 30 days and place the dishes under the same culture conditions.

After about 90 days from the beginning embryogenic proliferations arise, mainly from the inner integuments (Fig. 2c), at frequencies slightly higher than 1%. However, SE can be induced in at least half of the tested genotypes. To test the genetic fidelity of each embryogenic line with their respective donor tree is compulsory, because developing ovules are complex explants and not all embryogenic lines obtained from them are true-to-type. In fact, not only the obtaining of a line with the same genotype of the donor tree, but also lines that are the product of gametic fusion and even a line of gynogenic origin have been verified using microsatellites (Hernández et al., in preparation).

2.5 Induction of Somatic Embryogenesis in Apexes and Expanding Leaves from In Vitro Cultured Shoots

2.5.1 Embryogenic Culture Initiation

Axillary shoot cultures are used as the source of explants for initiating somatic embryos. Shoots are cultured in vertical position in 500-mL glass jars containing 70 mL of BM1 basal medium supplemented with cytokinin. They are transferred to fresh medium every 2 weeks with the following sequence: 0.44 μM BA for the first 2 weeks, 0.22 μM BA for the next 2 weeks, and 0.044 μM BA plus 0.45 μM zeatin for the last 2 weeks, in a 6-week multiplication cycle. Stock cultures are incubated in a growth chamber with a 16-h photoperiod (provided by cool-white fluorescent lamps at a photon flux density of 50–60 μmol m^{-2} s^{-1}) at 25 °C light/20 °C dark. The composition of the media mentioned below is described in Table 2.

1. Take shoot apices (2–2.5 mm long, comprising the apical meristem and the corresponding leaf primordia), and the most apical expanding leaf below the shoot apex from 6-week-old axillary shoot cultures (Fig. 3a, b).
2. Culture shoot apices (horizontally orientated) and leaf explants (abaxial side down) in 90-mm diameter Petri dishes filled with 25 mL embryogenic induction medium (M1). Ten apexes or ten leaf explants are placed per Petri dish.
3. Incubate the explants in darkness at 25 °C for 8 weeks.

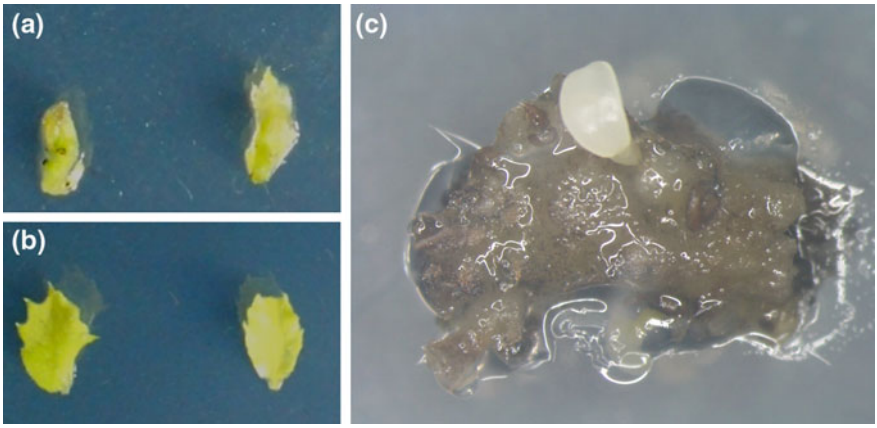

Fig. 3 Induction of somatic embryogenesis in explants from axillary shoot cultures established in vitro from holm oak trees. **a** and **b** Shoot apices and leaf explants used as initial explants, **c** somatic embryos initiated in a shoot apex explant

4. Transfer the explants into 90-mm diameter Petri dishes filled with 25 mL of expression medium (M2).
5. Incubate the explants in a growth chamber under the standard culture conditions for 4 weeks.
6. Transfer the explants onto expression medium (M3) in Petri dishes of the same type.
7. Incubate the explants in a growth chamber under standard conditions for 12 weeks.

Somatic embryos can be observed as early as 3 months after the start of experiments (Fig. 3c).

2.5.2 Histological Analysis to Confirm the Embryogenic Nature

The double staining safranin-fast green (Jensen 1962) or periodic acid-Schiff (PAS)-naphtol blue-black (Feder and O´Brien 1968) are used to confirm the presence of somatic embryos or embryogenic structures in the callus developed from the explants. Explants are processed according to Jensen (1962).

1. Fix the samples in a FAA solution (formalin, glacial acetic acid and 50% ethanol (1:1:18, v/v/v) for 48 h.
2. Dehydrate the samples through a graded n-butanol series, 48 h each.
3. Embed the dehydrated samples in paraffin wax.
4. Obtain serial sections of tissues of 8 μm thickness using a Reichert-Jung rotatory microtome.

5. Double-stain the sections with safranin-fast green or with PAS-naphtol blue-black.
6. Mount the stained sections with Eukit® and examine them using a transmission light microscope.

The embryogenic character of the embryogenic structures can be confirmed by the presence of meristematic cells, which should have a dense protein-rich cytoplasm, small vacuoles and a high nucleoplasmic ratio. Somatic embryos exhibit well-formed shoot and root meristems and closed vascular tissue.

2.6 Proliferation of Embryogenic Lines

As in other *Quercus* species, embryogenic lines of holm oak can be maintained proliferating by secondary embryogenesis on culture medium lacking plant growth regulators.

1. Pick up the nodular embryogenic structures and embryos at early stage of development showing secondary embryogenesis produced in the starting explants.
2. Culture them in Petri dishes with 25 mL of embryo proliferation medium, or alternatively in 100-mL baby food jars (Sigma-Aldrich, ref. V8630) filled with 40 mL of BM4 basal medium without PGR.
3. Place the cultures in a growth chamber at 25 °C in darkness or under a 16-h photoperiod provided by cool-white fluorescent lamps at a photon flux density of 50–60 μmol m^{-2} s^{-1}.
4. Subculture the white translucent proembryogenic masses or torpedo-stage embryos (Fig. 4a) onto fresh proliferation medium each 4–6 weeks, depending on the proliferation ability of each embryogenic line.

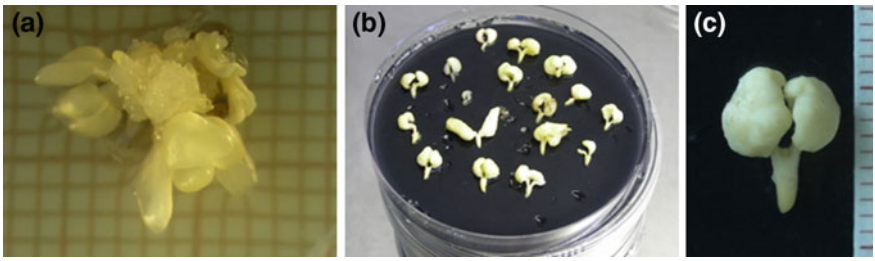

Fig. 4 Proliferation of embryogenic lines and maturation of holm oak somatic embryos. **a** Cluster of embryogenic nodules showing embryo differentiation, **b** isolated embryos after one month on maturation medium, **c** somatic embryo ready for germination

2.7 Embryo Germination and Plant Conversion

Cotyledonary-stage embryos (≥ 5 mm) without signs of secondary embryogenesis are the initial explants for this step. They can be spontaneously produced during proliferation of embryogenic lines and collected at each subculture time.

1. Pick up single cotyledonary embryos from the proliferating cultures and culture them in baby food jars with 40 mL of a maturation medium, or alternatively in 90-mm Petri dishes with 25 mL of maturation medium composed of BM5 basal medium 30 g/L sucrose, 0.02 g/L ascorbic acid, 10 g/L active charcoal and 6 g/L Plant Agar (Duchefa). (Fig. 4b). Alternatively maturation can be carried out on BM2 or BM4 basal media with macronutrients at half-strength, 30 g/L sucrose, 6 g/L Vitro agar (Pronadisa), supplemented or not with 60 g/L sorbitol.
2. Maintain these embryos at 25 °C in darkness for 1 month.
3. Cold store at 4 °C in darkness for 2 months.
4. Subculture white-opaque embryos of 10–15 mm in length (Fig. 4c) into plastic vessels (Sterivent High Container, Duchefa, ref. S1686. 15 embryos per vessel) filled with 500 mL of germination medium composed of BM4 basal medium, 30 g/L sucrose, 6 g/L Plantagar S1000 and lacking PGR.
5. Place these vessels in a growth chamber at 25 °C under a 16-h photoperiod (120–180 μmol m^{-2} s^{-1}) for 2 months.
6. Pick up the converted embryos (Fig. 5a) from in vitro vessels, wash them thoroughly with distilled water to eliminate all remains of culture medium and transplant to forest containers filled with substrate (peat:coconut fiber:perlite, 2:2:1, v/v/v) plus the 3 g/L of the slow-release fertilizer Osmocote™. Water with 2 mL/L Previcur®.
7. Place the containers within plastic boxes covered with plastic film in a growth chamber (Fig. 5b) with a 16-h photoperiod provided by mixed Sylvania Gro-Lux® and Philips cool-white fluorescent tubes (120–180 μmol m^{-2} s^{-1}) at

Fig. 5 Plant regeneration from somatic embryos of holm oak. **a** Single somatic embryo germinated and converted in a test tube, **b** plants transferred ex vitro for acclimatization, **c** single acclimatized plant

23 °C and 60% relative humidity. Spray the plants with a fungicide mixture (0.5 g/L Benoagrex®, 2 g/L Laincobre® and 1.5 g/L Captamur®) weekly.
8. After two weeks make a 1 cm aperture of the plastic film along one side of the box, and after one additional week a 1 cm aperture along the opposite side. From the second week onwards water as needed and weekly fertilize with a commercial fertilizer 20:20:20 at 0.5 g/L.
9. From the 4th week, open an additional 1 cm weekly on each side until the completion of 2 months. Then, move the surviving plants into the greenhouse.

Somatic embryos are reported to germinate on medium without PGR at rates between 53–85% and convert at rates between 11–30% depending on genotype. The supplementation of germination medium with 0.25 μM IBA plus 0.11 μM BA may slightly enhance conversion rates. Alternatively, cold stored somatic embryos can be germinated and converted in BM6 basal medium supplemented with 0.6 g/L Vitro agar, 30 g/L sucrose, 0.44 μM BA and 20 μM STS. Survival rates after acclimatization are very low.

2.8 Cryopreservation of Embryogenic Lines

Successful cryopreservation of holm oak tissues has been reported using a vitrification procedure. Initial explants are globular clusters collected from proliferating embryogenic lines.

1. Collect initial explants and culture on BM4 basal medium (Table 1) but with the sucrose concentration increased to 0.3 M for 3 days.
2. Prepare the vitrification solution PVS2 by mixing 30% w/v glycerol, 15% w/v DMSO (Me_2SO) and 15% w/v ethylene glycol in liquid BM4 basal medium containing 0.4 M sucrose.
3. Incubate the explants in 2-mL cryovials (10 embryo clusters per vial) containing 1.8 mL of PVS2 vitrification solution at 24 °C for 30 min.
4. Resuspend the explants in 0.6 mL of fresh PVS2 and place the cryovials into a cryogenic container with liquid nitrogen.
5. For thawing after cryostorage quickly plunge cryovials into a water bath at 40 °C for 2 min, and then drain off the PVS2 solution.
6. Wash the explants with liquid BM4 basal medium supplemented with 1.2 M sucrose, with two changes of medium each for 10 min.
7. Place the explants on filter paper discs and culture in 90 mm diameter Petri dishes filled with 25 mL of a proliferation medium composed of BM4 basal medium, 30 g/L sucrose, 6 g/L Plantagar S1000 and lacking PGR for 24 h.
8. Subculture the embryogenic masses onto the same fresh medium for growth resumption and recovery of the embryogenic line by secondary embryogenesis.

Growth recuperation of all cryopreserved holm oak embryogenic lines has been reported, but the recovery of somatic embryo differentiation ability is largely dependent on genotype.

3 Research Prospects

Although successful induction of SE in tissues from adult holm oak trees has been achieved, several bottlenecks in this plant regeneration system still remain. The most useful starting explants are shoot apexes and expanding leaves from in vitro axillary bud cultures, because this kind of cultures can be initiated at any time of the year and they are genetically homogeneous. Hence the somatic embryos produced are clearly clonal copies of the donor tree. Thus the SE induction from floral tissues would be limited to those genotypes that were recalcitrant to the induction in apices or leaves. Currently the most serious bottleneck is the acclimatization of converted plants. Attempts using conventional acclimatization procedures largely failed, and plant survival rates are very low. Perhaps the episodic growth, which is characteristic of *Quercus* species (Favre and Juncker 1989) and particularly important in holm oak, is behind this important drawback.

The present protocols may be enough to produce the limited number of plants needed to establish the compulsory clonal tests to validate varieties that can be deployed in plantations. However, for establishing operational plantations mass propagations systems have to be available. The development of suspension cultures and the production of somatic embryos in bioreactors are the ways followed in other *Quercus* species (Jiménez et al. 2011, 2013; Mallón et al. 2012) and have also been initiated in holm oak (Barra Jiménez 2015).

In addition to the important role played by somatic embryogenesis in the regeneration of plants transformed with genes that codify for pathogenesis-related proteins (Corredoira et al. 2016), an interesting possibility of holm oak somatic embryogenesis to cope with *Phytophthora* infections has been recently proposed. The induction of an "epigenetic memory" (priming) by culturing developing somatic embryos with several elicitors might induce a memory of resistance against the pathogen in the regenerated plants (Arrillaga et al. 2017). All these issues require a lot of research work.

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Somatic Embryogenesis of Greek Fir (*Abies cephalonica* Loud.)

Jana Krajňáková and Hely Häggman

1 Introduction

Euro-Mediterranean firs (the genus *Abies* Mill.) belong to ecologically and commercially most important tree genera in Europe. Fir forests represent a major component of Central European, Alpine and Mediterranean mountain forests. Their distribution ranges from 6°W to 44°E in longitude, from 35°N to 52°N in latitude and from 135 to 2900 m in altitude (Krajňáková et al. 2014; Alizoti et al. 2011). Fir species are of high economic importance both for timber (construction wood, furniture, pulp production, fuel wood etc.) and for non-wood forest products. Thanks to their fragrance, colour, good form and exceptionally long leaf retention after being cut, most of the firs are used as ornamental trees and are grown in plantations for Christmas trees (e.g. *A. borisii-regis, A. cephalonica* and *A. nordmanniana*). This is true also for hybrids—the genus *Abies* was object of intensive hybridization studies, and several artificial hybrids, including *A. alba* × *A. cephalonica* were found promising and exceeded pure species in growth (Kormuťák and Vooková 2001; Kobliha et al. 2013).

Greek fir (*Abies cephalonica* Loudon) is an endemic species, medium-sized tree, expanding mainly in southern and central Greece, at altitudes of 800-1700 m, covering an area of 200,000 ha of productive and conservation forests (Papadopoulos 2016; Alizoti et al. 2011; Raftoyannis et al. 2008). Mediterranean ecosystems are highly sensitive to climate change, as relatively minor decreases in rainfall and increases in temperature may lead to the expansion of adjacent

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semi-arid and arid ecosystems at the expense of Mediterranean ecosystems (Muscolo et al 2017; Chrysopolitou et al. 2013). Fir forests decline, noticeable mainly in southern and central Greece, is one of the earliest signs of climate change impact on the Greek forest ecosystems (Bank of Greece 2011). Considering a climatic scenario, it has been proposed that Mediterranean firs could be in danger in some parts of their present range, but, on the other hand, could also replace other species in more northern zones with temperate humid climates (e.g. silver fir, *Abies alba* Mill) (Aussenac 2002).

A lot of effort has been put into the development of vegetative propagation methods for firs (either classical or biotechnological approaches), in order to rapidly gain the benefits of traditional breeding to be utilized in reforestation or ex situ conservation strategies (reviewed by Krajňáková et al. 2014; Vooková and Kormuťák 2007, 2014). Among in vitro methods till now, only somatic embryogenesis proved to be promising and five European and Mediterranean species (*A. alba*, *A. cephalonica*, *A. cilicica*, *A. nordmania*, *A. numidica* and several hybrids) were regenerated (reviewed by Krajňáková et al. 2014; Vooková and Kormuťák 2014). Based on the success of regeneration method, the slow cooling cryopreservation protocols for *A. alba* (Krajňáková et al. 2013), *A. cephalonica* (Aronen et al. 1999; Krajňáková et al. 2011a, b), *A. nordmanianna* (Nørgaard et al. 1993; Misson et al. 2006) and their hybrids (Salaj et al. 2010) were developed (reviewed by Krajňáková et al. 2014).

In Greek fir, like in other conifers, the multi-step regeneration process of SE starts with the induction of pro-embryogenic masses, followed by somatic embryo formation, maturation, desiccation and plant regeneration (Fig. 1a–f). *Abies* species were among the first coniferous species where the induction of SE was reported (Erdelský and Barančok 1986a, b). However, a standard protocol for propagation by SE on a large scale is still lacking, the only exception is SE of *A. nordmanniana* in which case the technology has already been tested in large scale (Find 2016). *A. cephalonica* was regenerated by Krajňáková et al. (2008), and hybrid *A. alba* × *A. cephalonica* was regenerated by Salajová et al. (1996). Embryogenic cultures of *A. cephalonica* and hybrid *A. alba* × *A. cephalonica* have been derived from immature zygotic embryos (Krajňáková et al. 2008; Salajová et al. 1996). In case of hybrid (*A. alba* × *A. cephalonica*), initiation of embryogenic cultures was achieved when using also mature embryos (Salaj and Salaj 2003) and cotyledons derived either from seedlings or somatic embryos (secondary or repetitive SE) (Salajová and Salaj 2001).

Somatic embryogenesis of several *Abies* species, including *A. cephalonica*, differs from most of the other genera of the *Pinaceae*, because only cytokinin is needed for induction and proliferation (Nørgaard and Krogstrup 1995; Krajňáková et al. 2008). Maturation of Greek fir and hybrid *A. alba* × *A. cephalonica* somatic embryos is promoted by high concentrations of abscisic acid and usually maltose is the preferable carbohydrate. The addition of polyethylene glycol promoted the development of somatic embryos (Salaj and Salaj 2003; Krajňáková et al. 2009). For germination, well-developed cotyledonary somatic embryos are selected and subjected to a partial desiccation treatment for three weeks (Krajňáková et al. 2008, 2009).

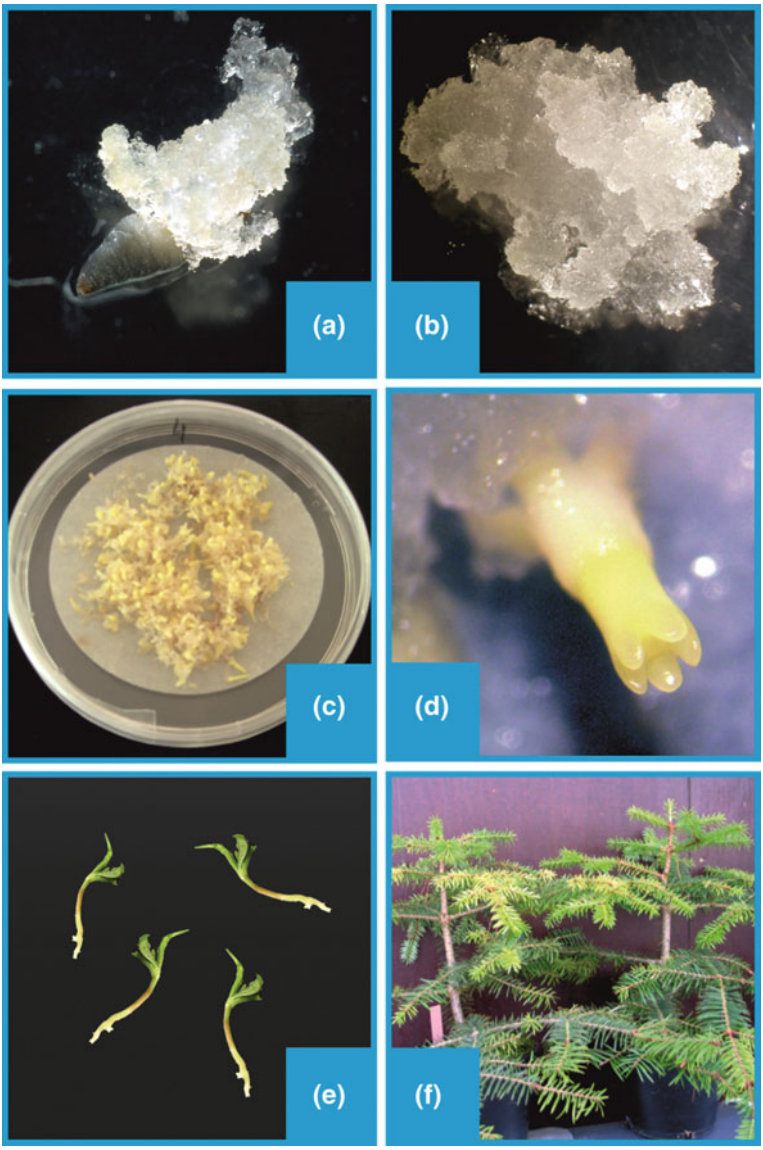

Fig. 1 Somatic embryogenesis of *Abies cephalonica*. **a** Initiation of somatic embryogenesis using immature embryos and proliferation of embryogenic cell mass, protruding from megagametophyte. **b**, **c** Proliferation of embryogenic cell masses. **d** Maturation of somatic embryos with embryogenic cell masses spread on filter paper. **e** Detail of cotyledonary somatic embryos. **f** Germinated somatic embryos. **g** Plants prepared for experimental field trail

Addition of the fulvic acids to the proliferation media has improved the proliferation abilities of Greek fir embryogenic cell masses, however have not had consequence on the further maturation process (Zancani et al. 2011). On the other

hand, co-cultivation of Greek fir embryogenic masses with ectomycorrhizal fungi reduced the proliferation of both tested embryogenic cell lines and led to improved maturation process (average number of cotyledonary somatic embryos produced per 1 g of embryogenic cell mass fresh weight) (Krajňáková et al. 2012).

Recently, the protocols for somatic embryogenesis of Greek fir and its hybrids were summarized by Krajňáková and Häggman (2016). In the present study we provide information about the best working protocol for regeneration of Greek fir by somatic embryogenesis that has been tested already in 3 independent laboratories. The slow-cooling cryopreservation protocol for embryogenic cell masses suitable for long-term storage is presented as well.

2 Materials

For starting each step of somatic embryogenesis, the general equipment for tissue culture work is required, e.g. laminar flow hood, scalpels, forceps, dissecting microscope, growth chamber or cultivation room, shaking incubators, autoclave, pH-meter.

2.1 Initiation, Induction and Proliferation

1. Seed cones of Greek fir containing immature seeds.
2. 70% ethanol, 4% (w/v) CaOCl (Ca-hypochlorite), sterile distilled water, 9-cm sterile Petri dishes, 100-mL sterile beaker.
3. Initiation medium (IM): solid MS-based medium (Murashige and Skoog 1962) with half-strength of macroelements supplemented with 20 g/L (58 mmol/L) sucrose, 1 mg/L (4.44 μmol/L) benzyl adenine (BA), 500 mg/L (3.4 mM) L-glutamine, and solidified with 0.3% (w/v) gellan gum Phytagel™ (Sigma) (Table 1)
4. Proliferation medium (PM): MS-based initiation medium (IM) with addition of 0.1% (*w/v*) casein hydrolysate (Table 1).

2.2 Maturation

1. Sterile filter paper discs, Falcon tubes, 9-cm sterile Petri dishes.
2. Liquid proliferation media (LPM) without plant growth regulators (Table 1) for making suspension with embryogenic cell masses (ECMs).
3. Maturation solid MS media: (i) half-strength macronutrient MS medium with 17 mg/L (64 μM) ABA, 10% (w/v) polyethylene glycol (PEG) 4000, 0.05%

Table 1 Composition of the initiation (IM), proliferation (PM), maturation (MM), and germination (GM) media during cultivation of Greek fir embryogenic cultures

Medium composition	Initiation (IM)	Proliferation (PM)	Liquid medium for suspension (LPM)	Maturation		Germination
Inorganics and organics	1/2-strength macro-elements MS	1/2-strength macro-elements MS	1/2-strength macro-elements MS	1/2-strength macro elements MS (MM1)	1/2-strength macro elements MS (MM2)	1/2-strength MS (GM)
Casein hydrolysate (g/L)		1	1	0.5	0.5	
L-glutamine		0.5	0.5	0.25	0.25	
Sucrose (g/L)	20	20	20			
Maltose (g/L)				30	30	20
BA (mg/L)	1					
ABA (mg/L)				17	8.5	
Phytagel (g/L)	3	3		2.5	2.5	
Agar (g/L)						10
PEG 4000 (g/L)				10		

(w/v) casein hydrolysate, 250 mg/L (1.7 nmol/L) L-glutamine, 30 g/L (83.3 mM maltose) (maturation medium 1, MM1); (ii) half-strength macronutrient MS medium with 8.5 mg/L (32 μM) ABA, 0.05% (w/v) casein hydrolysate, 250 mg/L (1.7 nmol/L) L-glutamine, 30 g/L (87.6 mM) maltose (maturation medium 2, MM2) (Table 1).

2.3 Germination, Conversion and Acclimatization to Ex Vitro

1. 150-mL tissue culture jars.
2. Solid half-strength MS hormone free medium with 20 g/l (58 nmol/L) sucrose, (germination medium, GM) (Table 1).
3. Non-fertilized horticultural peat and perlite.
4. Plastic containers, commercial fertilized peat (VAPO, Finland) with 1 kg/L basic fertilizer: 9.7% N, 7.5% P, 14.4% K, 5.0% Ca, 6.6% S, 3.8% Mg, 0.27% Fe, 0.13% Mn, 0.04% B, 0.05% Zn, 0.25% Cu, and 0.09% Mo and 3 kg/L limestone dust with Mg, commercial 0.2% 5-Superex fertilizer (Kekkilä, Finland).

2.4 Cryopreservation

1. Cryovials and markers.
2. Cryobox or cryocanes for immersion of the cryovials in liquid nitrogen (LN).
3. Sterile tips for pipets of different volumes (0.2 μL–1 mL).
4. Programmable controlled-temperature chamber or Nalgene™ freezing container and isopropanol.
5. Ice.
6. Dewar for the conservation of samples in LN.
7. Actively proliferating embryogenic cell masses (10–12 day old, after the last regular transfer).
8. Solid MS based medium for pre-treatment before cryopreservation: (i) half-strength macroelements MS medium, hormone-free, containing 68.5 g/L (0.2 M) sucrose (pre-treatment medium 1, PTM1); (ii) half-strength macroelements MS medium, hormone-free, containing 137 g/L (0.4 M) sucrose (pre-treatment medium 2, PTM2) (Krajňáková et al. 2011a, 2013) (Table 2).
9. Liquid MS based medium for cryopreservation: half-strength macroelements MS medium, hormone-free, containing 137 g/L (0.4 M) sucrose (cryopreservation medium, CM) (Table 2)

Table 2 Composition of proliferation (PM) and pre-treatments media (PTM) for cryopreservation of Greek fir embryogenic cell masses

Medium composition	Proliferation	Pre-treatment		Cryo-treatment
Inorganics and organics	1/2-strength macro-elements MS (PM)	1/2-strength macro-elements MS (PTM1)	1/2-strength macro-elements MS (PTM2)	1/2-strength macro-elements MS (CTM)
Casein hydrolysate (g/L)	1	0.5	0.5	1
L-glutamine	0.5	0.25	0.25	0.5
Sucrose (g/L)	20	68.5	137	137
BA (mg/L)	1	1	1	
Phytagel (g/L)	3	3	3	

10. Solid MS based proliferation medium (PM): half-strength macroelements MS medium with 20 g/L (58.4 mM) sucrose, 1 mg/L (4.44 µmol/L) BA, 500 mg/L (3.4 mM) L-glutamine, 0.1% (W/v) casein hydrolysate (Table 2).
11. PGD cryoprotectant solution: 10% PEG 6000, 10% glucose, 10% dimethyl sulfoxide (DMSO) in H_2O, filter sterilized.

3 Methods

3.1 Culture Media Preparation

1. Solid MS-based media for initiation (IM), proliferation (PM), and maturation (MM1 and MM2), are prepared in 9-cm Petri dishes and liquid media (LPM) to arrest proliferation in 250-ml Erlenmeyer flasks (Table 1). The germination medium (GM) (Table 1) is prepared into Magenta vessels. The pH of medium is adjusted to 5.7 prior adding the solidifying agent. Aqueous stock solutions of L-glutamine and ABA are filter sterilized and added to the medium after autoclaving. Separately autoclaved polyethylene glycol is mixed with the rest of the maturation medium in a laminar flow hood to get the final volume.

3.2 Embryogenic Culture Initiation

1. Immature zygotic embryos surrounded by the megagametophyte (called immature zygotic embryos) and isolated from immature seed cone are most favorable material for initiation SE of Greek fir. The optimum developmental stage of immature zygotic embryos for initiation is the precotyledonary stage (i.e., 1 month

after fertilization but before the formation of cotyledons). The cones with immature zygotic embryos can be stored at 4 °C for at least 2 months without losing the ability to induce somatic embryogenesis (Krajňáková et al. 2008).
2. Immature seed cones are rinsed with 70% ethanol for 2 min, after which the immature seeds are removed from the cones using scalpels and forceps and placed in a sterile beaker with sterile distilled water.
3. Seeds are surfaced sterilized for 20 min in 4% (w/v) CaOCl, and rinsed 3 times 5 min with sterile distilled water.
4. Seed coats are opened and removed with forceps and immature zygotic embryos surrounded by megagametophytes are excised and placed onto MS medium for initiation (IM).
5. Immature zygotic embryos are first cultured for 4 weeks, and thereafter transferred onto new media for an additional 4 weeks. However, it is recommended to control the contamination problems within the 1st week of cultivation. The contaminated immature embryos should be discarded and the not contaminated ones transferred onto a fresh medium.
6. Initiation and induction is performed in the dark at 22 ± 2 °C.

3.3 *Proliferation and Maintenance of Embryogenic Cell Masses*

1. Embryogenic cell masses start to protrude from different parts (micropylar end being the most frequent) of the responsive explants (immature zygotic embryos, surrounded by megagametophytes) 4-6 weeks after initiation (Fig. 1a). Embryogenic tissues are excised from each explant separately (each one representing one genotype) and transferred to a new Petri dish with proliferation medium (PM) to form a new cell line.
   To maintain the proliferation of ECMs, they are transferred to a fresh medium every 3 weeks. ECMs can be subcultured for several months (Fig. 1b) in the dark at 22 ± 2 °C. However, during prolonged proliferation, the regeneration ability of ECMs decreases. It is therefore very important to cryopreserved ECMs when stable proliferation is achieved.
2. Actively proliferating ECMs can be used for subsequent step, maturation.

3.4 *Maturation of Embryogenic Cultures*

1. ECMs are transferred to a maturation medium (MM1) 2 weeks after the last proliferation sub-culture. At the beginning of the maturation, 4 g of fresh ECM is transferred to sterile Falcon flasks with 20 mL of liquid hormone-free

proliferation medium (LPM) (Table 1). Suspension is gently mixed by vortex and allowed to settle. After the removal of supernatant, 1 mL of suspension, containing approximately 250 mg ECM (fresh weight), is plated onto sterile Whatman filter paper placed on MM1 medium (Table 1) for the first 6 weeks, followed by regular transfers to fresh media at 2-week intervals (Fig. 1c).
2. For further development of somatic embryos, ABA concentration is decreased and PEG-4000 is omitted from the medium, filter papers are transferred on maturation medium MM2 (Table 1).
3. Maturation is performed in the dark at 22 ± 2 °C and last 10–12 weeks.

3.5 *Desiccation-Conversion of Somatic Embryos and Acclimatization to Ex Vitro*

1. Mature healthy cotyledonary somatic embryos are carefully detached from the embryogenic cell masses (Fig. 1d) and transferred on empty Petri plates (diameter cca 4 cm), which are placed into bigger Petri plates (diameter cca 9 cm) with sterile distilled water for a 3 week desiccation period.
2. During the desiccation period, somatic embryos are stored in the dark, at the temperature of 4 °C.
3. Afterwards, desiccated embryos are placed onto the hormone-free half-strength MS medium with 20 g/L (58 mM) sucrose, solidified with 1% (w/v) agar (GM) (Table 1), which is prepared in Magenta vessels.
4. The base of somatic embryos is gently inserted into the medium.
5. Somatic embryos are germinated at the temperature of 22 ± 2 °C and the light intensity is kept for the first 2 weeks at 30 μE m^{-2} s^{-1} (16 h photoperiod), and then gradually augmented up to 75 μE m^{-2} s^{-1}.
6. Somatic embryo-derived plantlets are carefully detached from the medium and roots are washed (Fig. 1e). Thereafter plantlets are planted into small plastic greenhouses containing non-fertilized horticultural peat and perlite (*v:v*) (2:1). For the first 2 weeks the plantlets are kept under mist in order to keep relative humidity at approximately 90%, after which the humidity is gradually decreased.
7. After 1 month the plantlets are transferred into bigger containers (diameter cca 5–6 cm) containing commercial fertilized peat (VAPO, Finland) in a greenhouse. During the growing season, plantlets are fertilized monthly with commercial 0.2% 5-Superex fertilizer (Kekkilä, Finland). Figure 1f represents 4-year old plants of Greek fir ready for field trail.

3.6 Cryopreservation by controlled-rate cooling

Cold hardening, pretreatments and cryostorage

1. Transfer actively proliferating ECMs, size 300 ± 50 mg, on MS-based proliferation medium (PM, Table 2) and cultivate at 5 °C in the dark for 14 days. This step of cold hardening has been omitted from cryo-preservation protocol without noticing the decrease in viability of cultures after thawing, however only two cell lines were tested (Krajňáková et al. 2011b).
2. After cold hardening, transfer the culture onto proliferation medium supplemented with 0.2 M sucrose (PTM1, Table 2) for 24 h, and afterwards onto 0.4 M sucrose medium (PTM2, Table 2) for another 24 h.
3. Transfer about 3–4 ECMs clumps into 400 μL of hormone-free proliferation medium (CTM, Table 2), which is added into 2 ml cryotubes on ice.
4. Add PGD cryoprotective solution drop wise over a period of 30 min to give a final concentration of 5%.
5. Leave the cryotubes to stand for 2 h on ice before freezing.
6. After finishing the cryoprotection phase, freeze the samples at a rate of 10 °C h^{-1} (0.17 °C min^{-1}) to the prefreezing temperature of −38 °C, using a programmable controlled-rate freezer. It is possible to use also Nalgene Freezing Container, filled with isopropanol alcohol, which was successfully used for cooling down the cryovials, instead of using a programmable controlled-rate freezer (Krajňáková et al. 2011b).
7. After reaching the terminal temperature, immerse the cryotubes containing samples in LN and store.

Thawing and recovery

1. Thaw the cryovials in a 37 °C water bath and then transfer them on ice.
2. Rinse the surfaces of cryovials with 70% ethanol. Pay attention to labeling.
3. Plate (dispense) the contents of the cryovials on an autoclaved filter paper disc, placed on proliferation medium in a 90 mm Petri dish with 0.4 M sucrose (PTM2, Table 2). Incubate cultures for 1 h.
4. After 1 h, transfer filter papers with suspensions onto fresh proliferation medium with 0.2 M sucrose (PTM1, Table 2) and incubate for 24 h in dark at 22 ± 2 °C.
5. After 24 h, transfer filter papers with suspensions on the proliferation medium (PM, Table 2).
6. Examine the viability of cells by staining the suspension culture with 0.5% FDA (fluorescein diacetate) and observe at the microscope under UV light.
7. Monitor cultures regularly and transfer them onto fresh proliferation medium at 2-week intervals. The most precise way of monitoring the new proliferation is by determining the proliferation ratio (w_0/w_i) in which w_i is the initial fresh weight of sample after thawing and w_0 is the weight at the time of subculturing, generally 2, 4 or 6 weeks after thawing (Krajňáková et al. 2011a, b).

8. After observing the recovery (i.e., new proliferation growth), transfer the embryogenic cell masses on fresh proliferation medium without filter paper disc.
9. Embryo maturation is established when proliferation of cryopreserved ECMs is comparable to non-cryopreserved cultures.

4 Identify Steps Required Further Protocol Modifications

Despite the positive achievements, the bottlenecks of *A. cephalonica* somatic embryogenesis, like in most conifers, are the low initiation rate, uneven and long-lasting maturation phase, low quality of obtained somatic embryos, problems in germination and rooting phases. The current protocols for regeneration have some limitations and have been applied only to a few embryogenic cell lines (Krajňáková et al. 2014). The promising improvements of maturation step have been observed by applying dual cultures of specific ectomycorrhizal fungi with embryogenic cell lines of *A. cephalonica* (Krajňáková et al. 2013). However, the degree of embryogenic cell line responses is highly dependent on the interaction of a specific cell line and the fungal strain. The development of a reliable procedure for somatic embryogenesis of Greek fir for practical purposes is still the task for future research.

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SE Fluidics System

Cyrus K. Aidun and Ulrika Egertsdotter

1 Introduction

Somatic embryogenesis (SE) offers a basis for scalable, automated technology suitable for large-scale production of clonal plants. The SE method is attractive biologically due to the developmental path of the somatic embryo closely resembling zygotic embryo development thus avoiding issues related to adventitious rooting and plagiotropic growth. Furthermore, long term storage of SE cultures allow for field testing in species where zygotic seeds are the starting material for SE cultures, e.g. conifers. Application of SE methods for industrial scale plant production has been limited due to the cost of labor involved with different steps of the SE process (Lelu-Walter et al. 2013). Here we describe a novel fluidics-based technology where the most labour intensive steps of the SE process have been automated (SE fluidics system; Aidun and Egertsdotter 2012).

The SE fluidics system was primarily developed for SE in Norway spruce (*Picea abies* (L.) Karst), a key commercial species for northern Europe. Norway spruce accounts for 42% of the total volume of productive forest land in Sweden (Swedish Statistical Yearbook of Forestry 2014). Norway spruce is mainly propagated by seeds and to a limited extent by cuttings. However, among other problems, influx of foreign pollen in conventional seed orchards results in reduced gains in seedlings

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from the breeding program. Furthermore, propagation through cuttings is limited by rooting problems and plagiotropism (Högberg et al. 1998). Somatic embryogenesis has since long been considered for industrial scale production of Norway spruce in Sweden. With the recent innovations and development of an automated system for plant production, large-scale Norway spruce SE plant production is feasible (Aronen and Egertsdotter 2014).

Somatic embryogenesis in Norway spruce involves sequential in vitro steps concluded by plant regeneration and formation of a plant resembling a seedling from a zygotic seed embryo. Embryogenic cultures consisting of proembryogenic masses (PEMs) are initiated from zygotic embryos and multiplied on medium containing auxin and cytokinin. Withdrawal of these plant growth regulators prompts differentiation of mature somatic embryos from PEMs (Filonova et al. 2000). Subsequently, the mature somatic embryos are transferred to medium supplemented with abscisic acid (ABA) to induce somatic embryo maturation after which the somatic embryos are dried at high humidity and germinated into plantlets (Businge et al. 2012, 2013; Dobrowolska et al. 2017).

For scale-up and plant production, multiplication and maturation in petri plates are labour intensive steps that also results in large amounts of plastic waste. Here we use liquid medium in a versatile temporary-immersion bioreactor system for multiplication and maturation to reduce labor and waste (Businge et al. 2017). The subsequent harvest of mature embryos is the most labour intensive and prohibiting step for scale-up. Automation technology was developed for harvest of mature embryos and for the subsequent steps to planting of a germinated embryo ex vitro as described below (Aidun and Egertsdotter 2012).

2 SE Fluidics System for Automated SE Plant Production

2.1 Initiation and Storage

In conifers, somatic embryos can only be initiated from zygotic embryos where the zygotic embryo explant needs to be in a certain stage of early development for some species (e.g. *Pinus* sp, *Abies* sp.), whereas initiation can be from fully mature and stored seeds in many *Picea* species albeit at a lower initiation rate. Manual procedures where zygotic embryos are dissected from sterilized seeds and cultured on petri plates are still used for initiation of early stage somatic embryos forming callus-like, PEMs. When sufficient volume of PEMs has formed, the tissue can be cryopreserved and stored for prolonged time. A reliable method for cryopreservation is based on a simple pre-treatment of embryos followed by direct cryopreservation (Kong and von Aderkas 2011).

2.2 *Multiplication and Maturation*

(a) **Culture Dispersion**

Multiplication of PEMs results in clumps of connected tissue where the interior parts become dormant over time. To optimize multiplication rates and development of PEMs, it is beneficial to create a thin layer of PEMs where a larger number of early stage embryos are exposed to culture medium. Such growth conditions can be created by dispersing the clumps of PEMs into a suspension and distribute the suspension onto a supportive medium surface (Aidun and Egertsdotter 2015; Aidun 2015a). Increasing the proportion of active PEMs also increase the amount of mature embryos that can be formed, and thereby the conversion rate of mature embryos from PEMs. This procedure is particularly useful for culture in bioreactors based on liquid culture medium (Mamun et al. 2014).

(b) **Bioreactor Culture**

A temporary immersion bioreactor model was developed that meets the needs for easy-use and high-yield utilization for conifer somatic embryo multiplication and maturation (Fig. 1). This bioreactor can also be used for germination of conifer somatic embryos (data not shown), and shoot multiplication of hardwood trees (Businge et al. 2017). The bioreactor has separate containers for plant material and culture medium, allowing for easy and safe exchange of culture medium without disturbing the culture. For SE cultures of conifers, multiplication and maturation can occur sequentially in the same bioreactor without disturbing the culture. Bioreactors are started with PEMs cultures grown as suspension cultures, or solid cultures that are dispersed at start of the bioreactor culture.

Fig. 1 The new temporary immersion bioreactor-model developed primarily for conifer SE. Mature somatic embryos of Norway spruce in bioreactor (top view)

2.3 *Dispersion and Separation*

The mature embryos in a bioreactor are typically embedded ('glued') with the PEMs. In order to singulate and sort mature embryos for selection, it is necessary to disperse and separate mature embryos from PEMs. The sequence of processes in a SE fluidics system is shown in the flow chart of Fig. 2 (Aidun 2015b).

Typically, conifer SE cultures, on solid or in liquid medium, require these steps, as relatively large amount of PEMs is present during embryo maturation. There are two methods to accomplish dispersion/separation processes. The first method (Method A) is based on two steps, one for dispersion and the other for separation. The second (Method B) is a more advanced method where both dispersion and separation occur in the same device. Both systems are briefly explained below.

(a) **Method A—Individual**

With Method A, the disperser and separator are two different systems designed to disassociate the masses of embryos and embryogenic tissue from each other so that individual somatic embryos without any PEMs are collected and singulated in the system. Clusters of embryos embedded in PEMs are introduced from the bioreactor (or from solid medium in a petri plate) into the system. The disperser consists of tubes with varying cross-sectional area and constrictions. Such constrictions are comprised of different shapes and sizes whose radii consistently decrease. By the final cross-sectional change, the embryos and PEMs are completely disassociated and dispersed, but still mixed together.

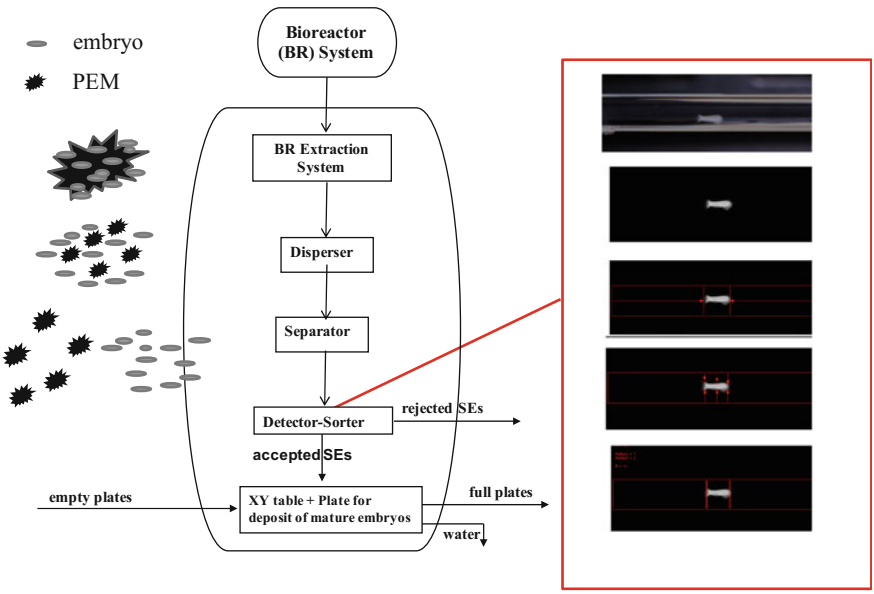


Fig. 2 Flow chart showing the various steps in the SE fluidics system from proliferation and maturation in bioreactor to extraction, dispersion, separation, image analysis, sorting and deposition

In the specific system outlined below, to separate all embryos from each other and the tissue between them, the mass is subjected to both radial and axial, extensional and compressional fluid dynamic forces of increasing magnitude. The rate of axial strain within the tubes is defined by $\frac{\partial u}{\partial z}$, where u_z is the fluid velocity in the axial direction, z, of the tube. This quantity imposes extensional strain when positive and compressional strain when negative. The dimensional constraints are periodically decreased in order to increase the hydrodynamic forces on the cluster of embryos-PEMs to gradually disassociate the mature embryos from the PEMs. However, the diameter of the passages must remain large enough to avoid damaging the embryos.

The separator subsystem is designed to separate somatic embryos from PEMs also traveling through the liquid after being dispersed. The result is that only embryos without any other material pass through the system for image analysis and deposition. The system is comprised of a cylindrical container with a device that rotates the fluid inside the container. At the bottom of the container there is a small conduit in the center of the base leading out of the section. The embryos having the same density as PEMs, but different drag coefficient, get into the conduit at the bottom as remaining PEMs stay floated and eventually leave the container through the side outlets.

At this point embryos are completely separated from PEMs. Both the separator and the disperser were tested on different tissue samples with great success, as shown below.

(b) **Method B—Integrated**

In the integrated method, the disperser and separator are in a single unit. The physical principle of operation and the device forming the separation are based on a different approach. However, the net result is the same; that is the embryos are separated from PEMs. In Method B, there is no moving parts, and the entire system works with the fluid pressure generated by a pump. The method is currently under development at Georgia Tech.

2.4 Orientation Control

The subsystem for embryo orientation (Aidun 2013) is designed to make sure that each embryo passing through the fluid flow tube is travelling in a specified orientation with the cotyledon crown either first or last. This system normally follows after the separator, however it can operate independently as well. As shown in Fig. 3, the system consists of a three-way junction where the fluid flow can be directed from **1** (IN) to either **2** (RESERVIOR) or **3** (OUT), depending on the stage of the process. The fluid flow can be directed from **1** to **2** or **3** as determined by the control unit. The tube that the fluid and embryos pass through is dimensioned small enough such that the embryos can only travel with their cotyledon crown first or

last. Prior to the three-way junction, there is an image analysis section where each embryo passing through is photographed and measured at several points to determine the orientation and if the somatic embryo is acceptable. The tube at the imaging section has a rectangular cross-section to allow photos to be taken through a flat glass surface rather than a rounded one to avoid image distortion.

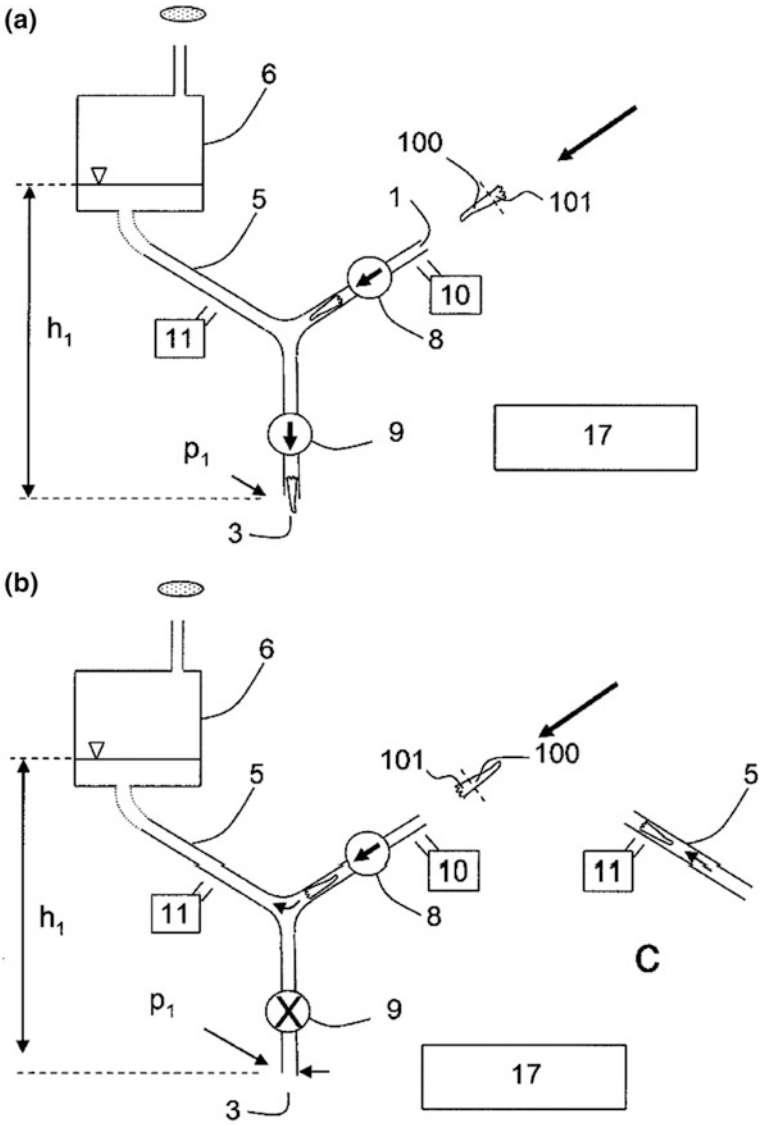


Fig. 3 Orientation correction subsystem; **a** embryo arriving with cotyledon last—correct orientation, **b** embryo arriving with cotyledon first—incorrect orientation

Orientation is first determined at the image analysis section prior to the three-way junction. Once the embryo is photographed, the control unit determines if the embryo is acceptable or not, and if acceptable, determines the orientation of the arriving embryo. If the embryo is oriented properly (cotyledon last) then the fluid flow is directed from 1 to 3, as shown in Fig. 3a. If the embryo is traveling with incorrect orientation (cotyledon first) then fluid flow is first directed from 1 to 2 up toward a reservoir, as shown in Fig. 3b, and then flow is directed from 2 to 3 and to the deposition section. The control unit regulates the direction of flow for either one of the steps. During the time that there is no embryo present, flow is directed from 1 to 3. After the somatic embryos pass through the orientation subsystem they continue onto the deposition section.

Not all SE fluidics systems require an orientation correction subsystem. If necessary, the orientation can also be corrected either after deposition or during germination and planting.

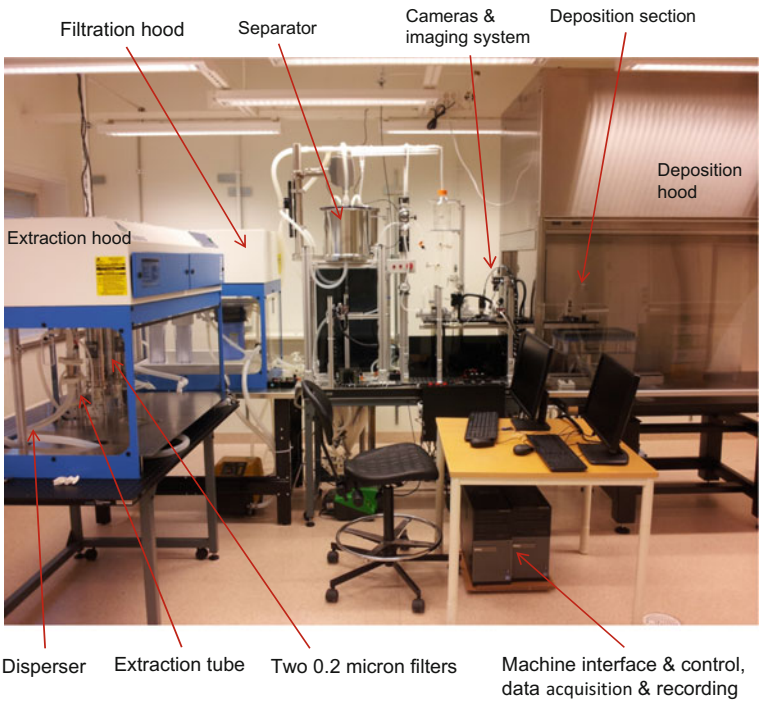
2.5 Imaging, Selection and Deposition

A major advantage of the SE fluidics system is the ability to image and tag or select embryos based on specific criteria. The process occurs as the embryo is passing through the deposition tube or prior to the orientation subsystem, if one is installed. The embryo triggers a laser sensor hardwired to the light source and cameras taking images from different angles. The image is then digitally analyzed in a matter of microseconds and results are evaluated based on pre-programmed criteria. If the embryo satisfies the required criteria, then it is deposited. Otherwise, the embryo is rejected. The imaging, image processing and decision making occurs in less that a millisecond therefore allowing continuous operation.

The decision to accept or reject an embryo depends on the selection criteria programmed by the user. The selection criteria are determined by the type of embryo and the specific characteristics associated with successful development for this particular embryo-type. It is also possible to use the system to establish the most effective selection criteria based on creating meta data and subsequent data mining and machine learning.

In many cases, simply the size of the embryo, straightness, shape or the number of cotyledons are good indicators of the maturity and quality of the embryo. Regular high resolution optical imaging from two or more angles can provide this information. If color, opacity or internal characteristics become parameters of importance, then other imaging methods can be used.

An SE fluidics system is shown in Fig. 4. This system is used for research and development at the Swedish University of Agricultural Sciences in Umeå, Sweden. There are typically three laminar hoods, at the Extraction, Filtration, and Deposition sections, to keep the system completely sterile. To start the system, water enters the main loop through the Extraction hood passing through two 0.2 micron filers in serial arrangement. Then the sterile water goes through the disperser (behind the



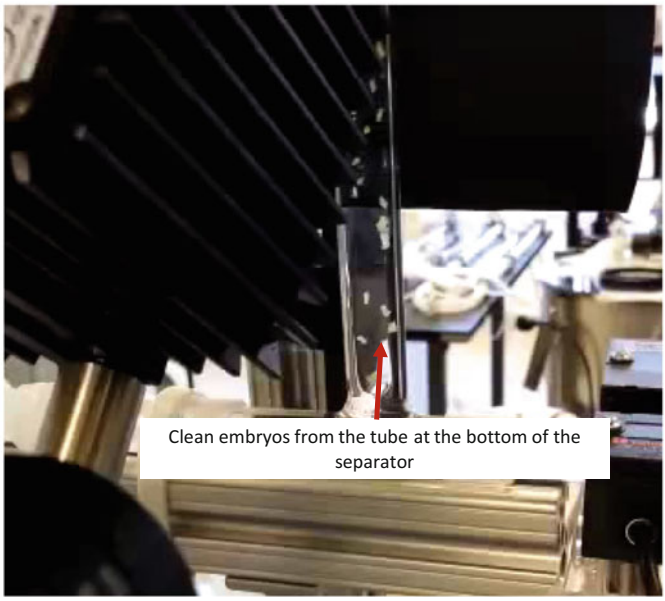


Fig. 4 Top—SE fluidics system located at Swedish University of Agricultural Sciences, Umeå, Sweden. Bottom—showing expanded view of the tube under the separator

Extraction hood), separator, imaging section, and finally the deposition section which is also under a hood. Once the system is operational, very small amount of water needs to be added as most of the water is recycled normally through a 150 micron filter first and then through 50 and 35 micron filters. Any fresh water entering the systems goes through the two 0.2 micron filters. The deposition hood is relatively large to allow various deposition trays and containers as well as actuation systems for continuous high throughput operations. The trays used require proper seal to remove from the hood.

3 Germination Platform and Planting

For smaller scale applications, mature embryos are deposited by the SE fluidics system directly into a partially immersed bioreactor or collected on a screen for subsequent culture steps; desiccation (if required), and germination. Once the root forms, the germinants can be handled in a non-sterile environment, therefore, it is easy to plant manually. However, for larger scale applications, automated planting is required.

(a) **Automated planting system**

In this section we describe the automated method for germination and planting developed at Georgia Tech (Aidun 2015c). The concept is outlined in Fig. 5 where a hollow tube is placed inside the substrate, the germinant goes inside the tube, and then the tube is withdrawn from the bottom leaving the germinant planted inside the substrate.

This simple approach has two main advantages over regular planting where the root is forcefully inserted into substrate (e.g., peatmoss mixtures). First the method of Fig. 5 results in better plant growth as shown in Table 1 where 135 germinants were planted with this method. The control is 135 identical germinants from Norway spruce planted manually in conventional way. The results show significant improvement. The quality rated "excellent" increased from (normally) 33% to 78% and the survivability increases from 81% to 95% (Aidun 2015c). The second advantage is that the approach allows large-scale automation (Aidun 2016).

(b) **Germination platform**

The germination platform (GP) described herein (Aidun 2016) is used for housing a plant propagule such as a somatic plant embryo, seed or other plant propagules from micropropagation through various necessary processes including but not limited to storage, desiccation, germination and planting. The invented GP is simple and inexpensive, and therefore, cost-effective for producing large numbers of quality-improved plants. It allows easy handling and transportation of the germinated propagule. Furthermore, because of the design, it produces more straight germinants and better quality roots with root hairs.

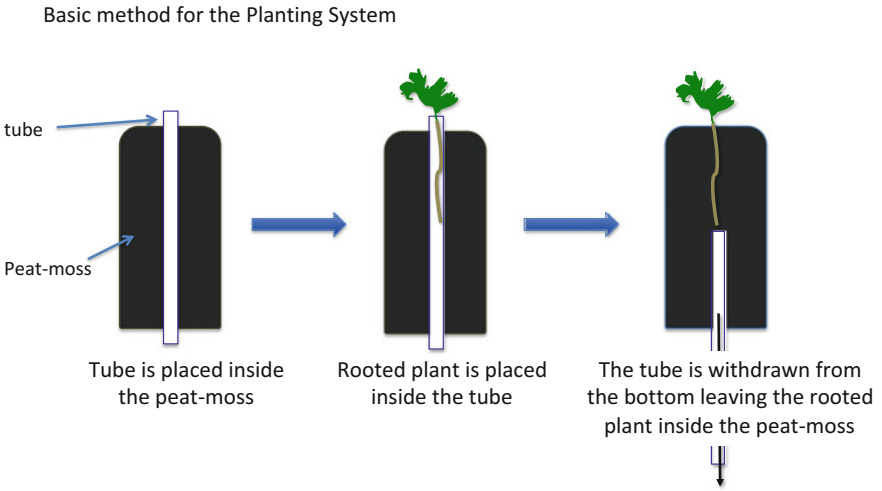


Fig. 5 Planting system based on Georgia Tech germination platform and planting approach (Aidun 2015c)

Table 1 Results from the GT planting approach

Norway spruce SE	Number planted (n)	Results excellent quality (% n)	Survival (% n)
Planted by hand	135	51 (33%)	110 (81%)
Planted with MPS	135	106 (78%)	128 (95%)

Aidun (2015c)

The key components of the GP are a circular paper disk and a circular tube. The embryo is placed at the center of the paper disk, then the paper disk is folded around the embryo such that the embryo is oriented longitudinally at the base of the folded paper disk, as shown in Fig. 6.

The folded paper disk containing the embryo is placed inside the tube such that the root-end of the embryo is pointing in the axial direction of the tube, and the tube placed inside a perforated plate. The perforated plate can hold from a few to few thousand tubes at one time.

The perforated plate is placed inside a sealed container, such that if necessary the entire assembly of tubes and paper disks can be kept sterile during desiccation, germination, and planting. One type of set up (for research and development) is shown in Fig. 7 where a partially immersed bioreactor is retrofitted to serve as a desiccation/germination box.

The advantage of the method is that the tube with the root inside can be coupled to an automated planting system. The coupling allows planting the germinant inside

Fig. 6 The key components of the GP. A paper disk folds over the SE and is placed inside the tube such that the root-end of the embryo is pointing in axial direction into the tube

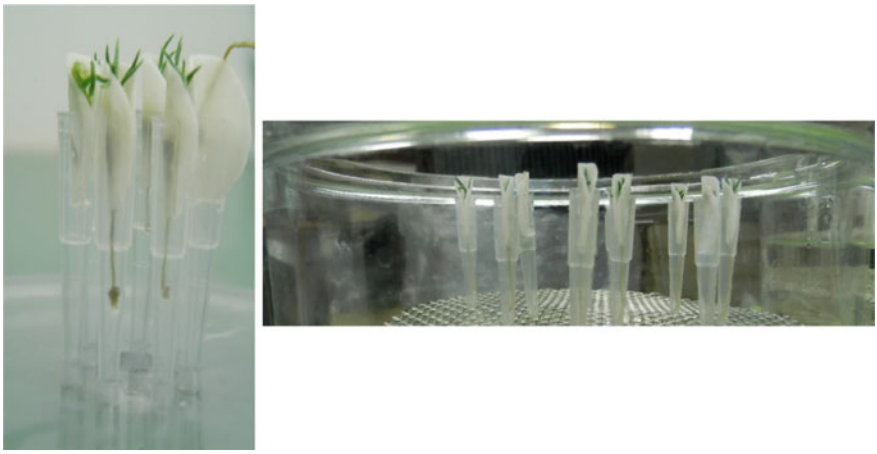


Fig. 7 Paper disks with embryos inside were placed inside tubes made from stereolithography. The tubes were placed inside a perforated plate inside a sealed container, as described above. Germination medium was added to the container so the paper disk and the embryo were soaked with the liquid germination medium. After two weeks, roots and shoots developed where the roots penetrated inside the tube

the substrate, such as peat moss. With the described germination platform, there is no need for a mechanism to place the roots inside the tube since the root is already growing inside a tube.

As outlined above, the embryo can be placed inside the paper disk, disk folded and placed inside the tube manually. Here, we describe a method for automatic preparation and placement of the folded disk with the embryo in the correct orientation inside the tube. The method and the device are described below.

The method is to place the SE flat on the middle of the paper disk. This could be done at the deposition section of the SE fluidics system, as described by Aidun (2015b, 2016), or any other method of depositing the embryo in the middle of the paper disk. Then the SE on the paper disk is imaged to determine the orientation in the plane of the paper disk. Once the orientation is determined, then the paper disk is rotated (e.g., with a rotary actuator) so the embryo orientation is always in a designated direction where the folding takes place. As the paper disk is folded (e.g., with a grip actuator), the embryo will have the root-end in a designated direction. The advantage of this method is that the root will grow inside the tube and therefore, it will be easy to handle, transport, store and plant. Because the germinant is already inside a tube, the planting can be done by any actuation system where the tube is picked and placed inside the substrate. One such system is explained by Aidun (2016) with a unit that would collect 135 tubes at a time and place inside a 9×15 tray.

(c) **SE fluidics system and synthetic seeds**

Significant progress has been made in development of synthetic seed concepts where a somatic embryo is housed inside a capsule with required nutrition for germination and growth (see review by Sharma et al. 2013). A synthetic seed would provide ease of storage, transportation and planting. For some cases, the capsule has an orientation and therefore the embryo must be deposited with the correct orientation inside the capsule. Combination of the automated SE fluidics system with orientation control outlined above with the synthetic seed concept has great potential.

4 Future Research Prospects

The SE fluidics system enables scale up of plant propagation by SE for industrial applications and for research. Efficient production of large numbers of embryos in bioreactors and automated embryo harvest offers opportunities to generate large sets of meta data that can be used to address fundamental research questions. Results from such studies will increase our basic understanding of the SE process and can be used to support optimization of methods and improve the overall production technology (Fig. 8).

Specifically, the SE fluidics system for research and development offers opportunities for different approaches through various modes of operation (Fig. 9). To obtain a description of the path of development for different embryo-types, the first mode of operation can be used. Individual embryos are tagged and followed

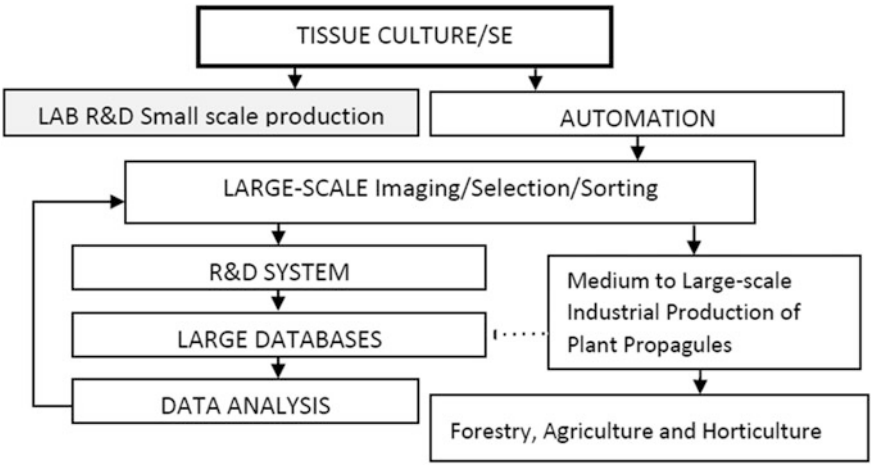


Fig. 8 Automation of tissue culture techniques allow for large scale production of plants and collection of large data sets. Analyses of meta data can support optimization and method improvements for both research and industrial applications

through development such that a direct correlation between embryo morphology and developmental capability is obtained. Understanding how embryo morphology correlates to germination and plant formation is key to increasing yields through improved selection criteria allowing harvest of the best embryos. The second mode of operation is used when the goal is to get high yields of embryos matching specific selection criteria. Based on information from studies using the first mode of operation, selection criteria for embryos can be refined for increasing yields of embryos that can develop to plants, as required for industrial production. Furthermore, selection criteria can also be set to identify embryo morphologies as specified by the research approach, that is, certain morpho-types resulting from transgenic modifications or previously described (by studies using the first mode of operation) and targeted for further studies. The imaging system can also be used for color detection such that embryos can be selected based also on color spectrum signature, as outlined below. In the third mode of operation, the system is used for fast and unspecific harvest of all embryos, doing a basic separation of mature embryos from PEMs. This is useful when the culture to be harvested already has a high yield of good embryos and selection of the best embryos do not significantly improve the yield.

The measurable characteristics showing developmental stage and quality of embryos with respect to germination and plant development depend on species and genotype. Various attributes such as size, shape, number of cotyledons, surface texture, color or internal structure could be directly correlated to the quality of the embryo with the highest yield and best quality plant. Depending on the specific

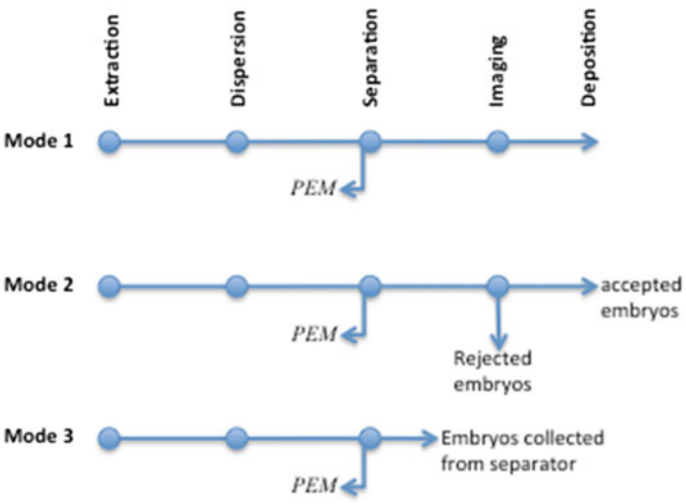


Fig. 9 Depending on the requirements, the system can be operated in three different modes: (1) all embryos are captured by image analysis, labelled and deposited; (2) certain embryos with specific characteristics are selected by image analysis, labelled and deposited; and (3) all embryos are harvested without image analysis or deposition

characteristics, various imaging methods can be retrofitted to the SE fluidics system to examine the most effective selection criteria. In general, there are three classes of imaging systems that can be implemented. These are regular RGB optical imaging, hyperspectral imaging, and internal imaging such as magnetic resonance imaging (MRI) or X-ray computed tomography (CT).

From regular high-speed/resolution optical imaging, many attributes can be automatically captured and analyzed at high speed through various angles recreating the three-dimensional embryo. Size, shape, aspect ratio, number of cotyledons and appearance of the embryo can be examined, compared and tagged at high speed.

Hyperspectral imaging (HSI) acquires a three-dimensional dataset called hypercube, with two spatial dimensions and one spectral dimension (Fig. 10). Spatially resolved spectral imaging obtained by HSI provides diagnostic information about the tissue physiology, morphology, and composition. In many species, the quality of the (embryo) tissue correlates directly with a specific optical wavelength. This approach has been used successfully with animal tissue and in some limited studies with plant tissue. Further application to characterize developmental stages and quality of plant embryos could prove most beneficial.

To examine internal characteristics of the embryo, MRI or CT scan can be applied. Contrast and speed of imaging are challenges that may limit these applications. However, in cases where the quality of the embryo depends on internal features, these imaging methods can be retrofitted to the SE fluidics system for non-intrusive sectioning and analysis of the embryo generating a wealth of information by examining and correlating internal features with developmental stages and quality of embryo for germination and plant development.

To conclude, different applications of the SE fluidics system for research and plant production support scale-up of embryo production and harvest. Coupled with specific detection technology, a broad field of applications focused on understanding developmental processes and increasing yields for crops in forestry, agriculture and horticulture can be envisioned.

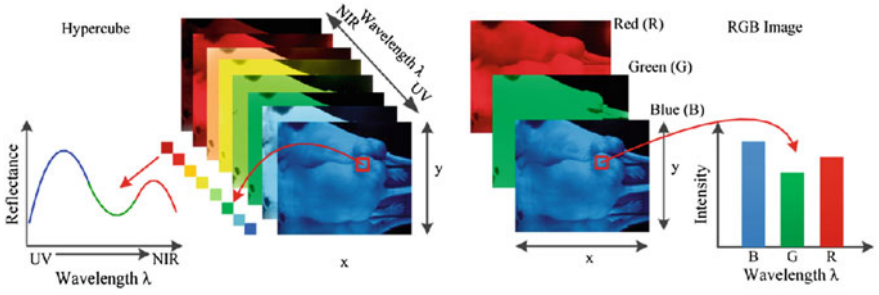


Fig. 10 Hypercube is a three-dimensional dataset of two-dimensional images at each wavelength. The lower left is the reflectance curve (spectral signature) of a pixel in the image. RGB color image only has three image bands on red, green, and blue wavelengths respectively. The lower right is the intensity curve of a pixel in the RGB image. From: Lu and Fei (2014)

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Protocol for Somatic Embryogenesis in Japanese Black Pine (*Pinus thunbergii* Parl.) and Japanese Red Pine (*Pinus densiflora* Sieb. *et* Zucc.)

Tsuyoshi E. Maruyama and Yoshihisa Hosoi

1 Introduction

Japanese black pine (*Pinus thunbergii* Parl.) and Japanese red pine (*P. densiflora* Zieb. *et* Zucc.) locally named "*kuromatsu*" and "*akamatsu*", respectively, are two important forest tree species widely used for reforestation and landscaping in Japan. In addition, *P. thunbergii* is also planted along coastal areas to prevent sand movement, erosion, and damage by salt spray, and *P. densiflora* is also important as host species of the very expensive "*matsutake*" mushroom, *Tricholoma matsutake* (Kosaka et al. 2001; Maruyama and Hosoi 2016a).

However, in recent years, Japanese pine populations have further declined as a result of pine wilt disease, caused by the pinewood nematode, *Bursaphelenchus xylophilus* (Kuroda 2004; Akiba and Nakamura 2005). Pine wilt disease is one of the most serious pests in Japan, and has been a key critical factor in the mass mortality of the Japanese pine forests (Kanetani et al. 2001; Kanzaki et al. 2011; Maruyama and Hosoi 2014). The nematode is inferred to be native to North America and since its introduction into Japan at the beginning of the 20th century, the pinewood nematode has spread to Korea, Taiwan, and China and has devastated pine forests in East Asia (Togashi and Shigesada 2006). It was also found in Portugal in 1999 (Mota et al. 1999).

Somatic embryogenesis is one of the most promising techniques for mass propagation of selected trees. It allows, the ex situ conservation of genetic resources by cryopreservation techniques, and for the purposes of genetic transformation (Park et al. 1998; Bonga 2016; Maruyama and Hosoi 2016b). Studies on somatic embryogenesis of Japanese pines has been reported (Ishii et al. 2001; Taniguchi 2001; Hosoi and Ishii 2001; Maruyama et al. 2005a, b, 2007; Shoji et al. 2006;

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Hosoi and Maruyama 2012; Kim and Moon 2014). In these studies, the low induction frequency of embryonal masses reported, reflect the arduousness in establishing embryogenic cultures in comparison with other Japanese conifers (Ogita et al. 1999; Maruyama et al. 2000, 2002, 2005c; Taniguchi and Kondo 2000; Igasaki et al. 2003; Taniguchi et al. 2004; Nakagawa et al. 2011; Maruyama and Hosoi 2012a; Hosoi and Maruyama 2016). In addition, although high somatic embryo maturation frequencies were observed in maturation media supplemented with polyethylene glycol, the achieved germination frequencies were relatively low (Maruyama et al. 2005a, b). Later, an improved protocol for somatic embryo germination of Japanese pines based on the desiccation of somatic embryos after the maturation on medium containing polyethylene glycol was reported (Maruyama and Hosoi 2012b). This post-maturation treatment markedly increased germination frequencies and considerably improved synchronization during the germination period similarly reported in other conifers (Roberts et al. 1990, 1991; Hay and Charest 1999; Klimaszewska and Cyr 2002; Stasolla and Yeung 2003; Klimaszewska et al. 2007).

The protocol described here is based on somatic embryogenesis initiated from immature seeds and plant regeneration obtained from somatic embryos after maturation on medium with polyethylene glycol. Procedures including explant preparation, embryonal mass induction and proliferation, somatic embryo maturation, plant conversion, and acclimatization are described.

2 Protocol for Somatic Embryogenesis in Japanese Black Pine and Japanese Red Pine

2.1 Culture Media

1. The culture media used for Japanese black pine and Japanese red pine somatic embryogenesis are described in Table 1.
2. Note that this protocol consists of several in vitro culture stages differing in medium, culture condition, and duration as described in Table 2.
3. Adjust medium to pH 5.8, and autoclave for 15 min at 121 °C and 1.1 kg cm^{-2}.
4. Amino acids stock solutions and abscisic acid (ABA) are filter sterilized and added to the medium after autoclaving.
5. Dispense media in culture vessels as specified in Table 2. Plates are sealed with Parafilm (Parafilm M® film, Bemis Company, Inc., Wisconsin, USA). Flasks are capped with transparent Tetoron film (Toray Ind., Tokyo, Japan).

Table 1 Constituents of culture media[a] for Japanese black pine and Japanese red pine plant regeneration system via somatic embryogenesis

Constituents	M1 (mg/L)	M2 (mg/L)	M3 (mg/L)	M4 (mg/L)	M5 (mg/L)
Basal salts					
KNO_3	500	500	1000	500	500
$NaNO_3$	30	30	60	30	30
$NaH_2PO_4{\cdot}2H_2O$	80	80	160	80	80
KH_2PO_4	35	35	70	35	35
$MgSO_4{\cdot}7H_2O$	250	250	500	250	250
$CaCl_2{\cdot}2H_2O$	37.5	37.5	75	37.5	37.5
$Ca(NO_3)_2{\cdot}4H_2O$	30	30	60	30	30
KCl	40	40	750	40	40
$MnSO_4{\cdot}4H_2O$	10	10	20	10	10
H_3BO_3	20	20	40	20	20
$ZnSO_4{\cdot}7H_2O$	12.5	12.5	25	12.5	12.5
KI	0.5	0.5	1	0.5	0.5
$CuSO_4{\cdot}5H_2O$	1.2	1.2	2.4	1.2	1.2
$Na_2MoO_4{\cdot}2H_2O$	0.1	0.1	0.2	0.1	0.1
$CoCl_2{\cdot}6H_2O$	0.1	0.1	0.2	0.1	0.1
$FeSO_4{\cdot}7H_2O$	15	15	30	15	15
Na_2-EDTA	20	20	40	20	20
Vitamins					
Myo-Inositol	500	500	1000	500	500
Thiamine hydrochloride	2.5	2.5	5	2.5	2.5
Pyridoxine hydrochloride	0.25	0.25	0.5	0.25	0.25
Nicotinic acid	2.5	2.5	5	2.5	2.5
Glycine	2.5	2.5	5	2.5	2.5
Plant growth regulators					
2,4-D	2.21	0.663			
BAP	1.125	0.225			
ABA			26.4		
Other additives					
Casein acid hydrolysate	500				
Glutamine	1000	1500	7300	400	
Asparagine			2100		
Arginine			700	250	
Citrulline			79		
Ornithine			76		
Lysine			55		
Alanine			40		
Proline			35	100	
Sucrose	10,000	30,000			30,000

(continued)

Table 1 (continued)

Constituents	M1 (mg/L)	M2 (mg/L)	M3 (mg/L)	M4 (mg/L)	M5 (mg/L)
Glucose				30,000	
Maltose			50,000		
Polyethylene glycol			100,000		
Activated charcoal			2000	2000	5000
Gellan gum	3000	3000	3000	6000	
Agar					11,500
pH	5.8	5.8	5.8	5.8	5.8

[a]M1 (Embryogenic culture initiation medium), M2 (Embryonal mass maintenance/proliferation medium), M3 (Somatic embryo maturation medium), M4 (Somatic embryo germination medium), M5 (Somatic plant growth medium)

2.2 Explant Preparation

1. Collect immature cones (Fig. 1a) from mother trees in mid-July.
2. Remove the seeds from the immature cones.
3. Disinfect excised seeds (Fig. 1b) with 1% (w/v available chlorine) sodium hypochlorite solution for 15 min and then rinse five times with sterile distilled water, 3 min each time.
4. Transfer the sterile seeds in a sterile plate.
5. Remove the seed coat with sterile scalpel and forceps and aseptically isolate megagametophytes from the seeds under a dissecting microscope.

2.3 Embryogenic Culture Initiation

1. For induction of embryonal masses, put horizontally the isolated megagametophyte explants on the surface of initiation medium (M1, Table 1) contained in Quad-plates (three explant per well, twelve per plate).
2. Seal culture plates with Parafilm and incubate under conditions described in Table 2.
3. The presence (Fig. 2) or absence of distinct early stages of somatic embryos characterized by an embryonal head (dense cells) with suspensor system (elongated cells) from the explant is observed weekly under the inverted microscope, up to 3 months. Initiation of embryonal masses is recorded if distinct early stages of somatic embryos proliferated after the first subculture.

Table 2 Medium, culture conditions, and culture durations for each stage of somatic embryogenesis in Japanese black pine and Japanese red pine

Stage	Medium[a]	Culture conditions[b]	Duration (week)
1. Embryogenic culture initiation	M1	Dark, 90 × 15 mm quad-plates (30–35 ml medium/plate) 3 megagametophytes/well (12/plate)	4–12
2. Embryonal mass maintenance/ proliferation	M2 or M2[c]	Dark, 90 × 15 mm quad-plates (30–35 ml medium/plate) 12 embryonal masses/plate Dark, 100 ml flasks, 60–80 rpm (50 ml medium/flask)	2–3 1–2
3. Somatic embryo maturation	M3	Dark, 90 × 20 mm mono-plates (30–40 ml medium/plate) 5 embryonal masses/plate	6–8
4. Somatic embryo desiccation	Filter paper	Dark, six-well multiplates (20–30 somatic embryos/well)	2–3
5. Somatic embryo germination	M4	Light, 90 × 20 mm mono-plates (30–40 ml medium/plate)	2–4
6. In vitro growth of somatic plants	M5 or Vermiculite	Light, 300 ml flasks (100 ml medium/flask) or (100 ml vermiculite/flask irrigated with a 0.1% (v/v) hyponex solution[d])	8–12
7. Ex vitro acclimatization of somatic plants	Vermiculite or Kanuma soil	Light, plants inside a growth chamber at 25/20 ± 1 °C and 80 ± 5% relative humidity were irrigated with a 0.1% (v/v) Hyponex solution[d]	3–5

[a]See Table 1
[b]Culture at 16-h photoperiod (65 µmol m^{-2} s^{-1}) or darkness at 25 ± 1 °C
[c]M2 medium without gellan gum
[d]Hyponex 6-10-5 plant-food solution (The Hyponex Co., Inc., Hyponex Japan, Osaka, Japan)

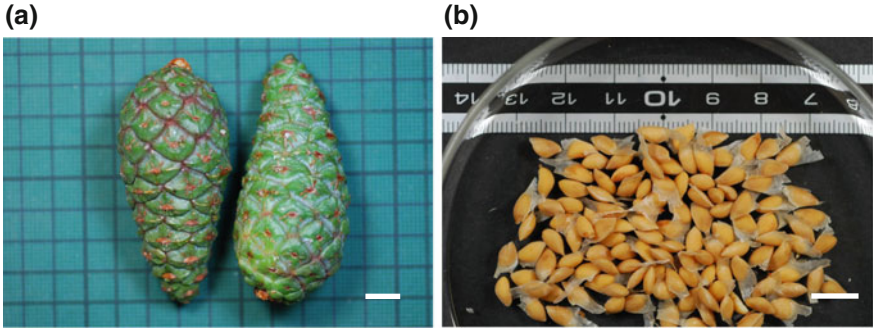

Fig. 1 **a** Immature cones of Japanese black pine, **b** excised seeds from the cones. *Bars* 1 cm

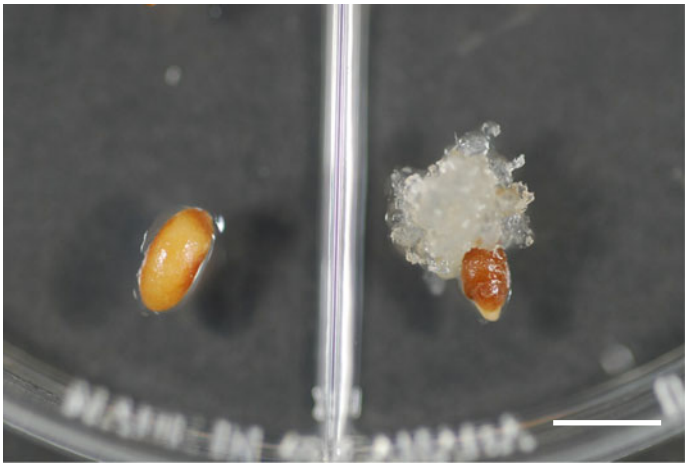

Fig. 2 Induction of embryonal mass from megagametophyte explant of Japanese black pine. *Bar*: 1 cm

2.4 Maintenance and Proliferation of Embryonal Masses

1. Collect embryonal masses from initiation medium with forceps and transfer to maintenance/proliferation medium (M2, Table 1). Seal plates with Parafilm and culture as describe in Table 2.
2. For subsequently maintenance/proliferation routines (Fig. 3), transfer twelve embryonal masses (about 20 mg FW each) onto M2 medium (three masses per well, twelve per plate) and subculture at 2 to 3-week intervals.

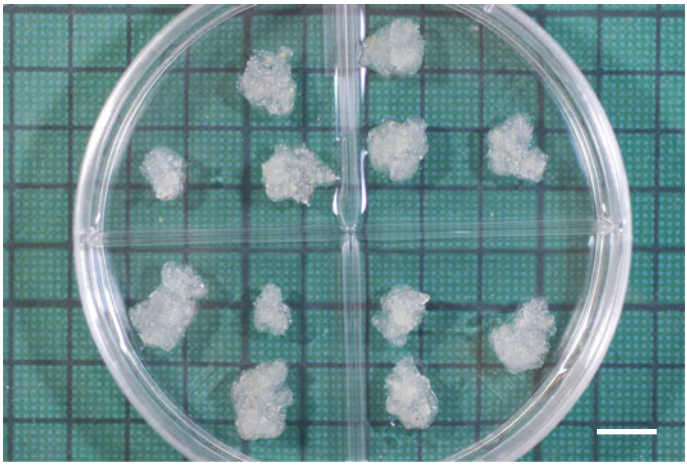

Fig. 3 Maintenance and proliferation of embryonal masses of Japanese black pine. *Bar*: 1 cm

3. As an alternative method for more rapid proliferation making of cell suspension cultures, transfer embryonal masses (about 50 mg FW) to 100 ml flasks containing 50 ml liquid medium (M2 medium without gellan gum) and culture in rotary shaker with shaking at 60–80 rpm in darkness at 25 ± 1 °C. For continuously proliferation routines in liquid medium, subculture embryonal masses to same fresh medium (about 0.5 ml suspension culture in 50 ml fresh medium) at 1 to 2-week intervals.

2.5 *Maturation of Somatic Embryos*

1. Collect proliferated embryonal masses from maintenance/proliferation medium with forceps and transfer five masses (about 100 mg FW each) onto plate containing maturation medium (M3, Table 1). Homogeneously disperse each embryonal mass with forceps on a surface equivalent to a circle of about 2.5–3 cm in diameter. Seal plates with Parafilm and culture as describe in Table 2.
2. For suspension cultures, collect embryonal masses on 100 µm nylon screen and rinse embryonal masses with M2 medium without plant growth regulators and gellan gum. Resuspend embryonal masses in same medium (about 500 mg FW in 2.5–3 ml medium) and homogeneously disperse with pipettes onto 70-mm-diameter filter paper disks over each plate containing maturation medium (M3, Table 1). Seal plates with Parafilm and culture as describe in Table 2.
3. Initial formation of cotyledonary embryos are observed about 4 week after transfer of embryonal masses to the maturation medium, and is evident at 6–8 week of culture (Fig. 4).

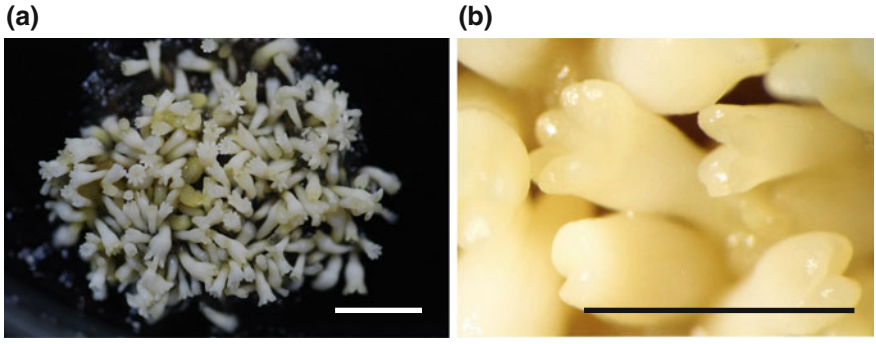

Fig. 4 Cotyledonary embryo formation in Japanese black pine (**a**) and Japanese red pine (**b**). *Bars* 1 cm

2.6 *Somatic Embryo Desiccation*

1. Collect cotyledonary embryos from maturation medium and transfer onto 30-mm-diameter filter paper disks over plate containing germination medium (M4, Table 1).
2. Transfer the embryos placed over 30-mm-diameter filter paper disks into 2 (central) wells of a Six-well multiplate in which the remaining 4 (side) wells are filled with about 5 ml of sterile water (Fig. 5).
3. Seal plates with Parafilm and incubate as describe in Table 2.

2.7 *Germination of Somatic Embryos*

1. Collect filter paper disks containing desiccated somatic embryos and transfer onto plates containing germination medium (M4, Table 1).
2. Seal plates with Parafilm and culture as describe in Table 2.
3. About 2–4 week after transfer to germination medium germinated embryos (Fig. 6) can be transferred to growth medium for plant conversion.

2.8 In Vitro *Growth of Somatic Plants*

1. Collect germinated somatic embryos from germination medium and transfer to flasks containing growth medium (M5, Table 1; Fig. 7a). Flasks are capped with transparent Tetoron film and culture as described in Table 2.

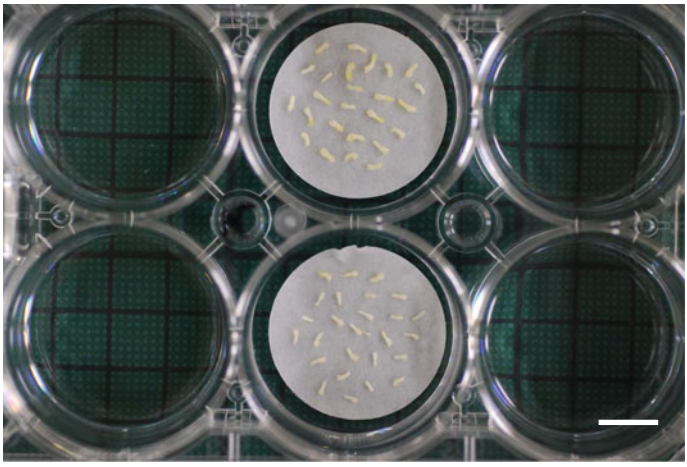

Fig. 5 Desiccation of somatic embryos in six-well multiplate at high relative humidity. *Bar* 1 cm

Fig. 6 Germination of somatic embryos after desiccation treatment. *Bar* 1 cm

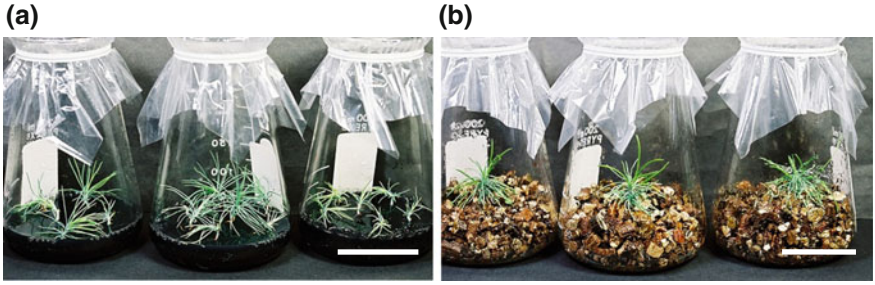

Fig. 7 In vitro growth of somatic plants after transfer to flasks containing M5 medium (**a**) or Vermiculite with Hyponex nutrient solution (**b**). *Bars* 5 cm

2. As an alternative method, transfer germinated somatic embryos to flasks containing Vermiculite with Hyponex nutrient solution and culture as described in Table 2 (Fig. 7b).
3. About 8–12 week after transfer to growth medium somatic plants can be transferred to ex vitro conditions for acclimatization.

2.9 Ex Vitro *Acclimatization and Field Transfer*

1. Remove somatic plants from culture flasks and transplant into plastic pots filled with vermiculite or Kanuma soil.

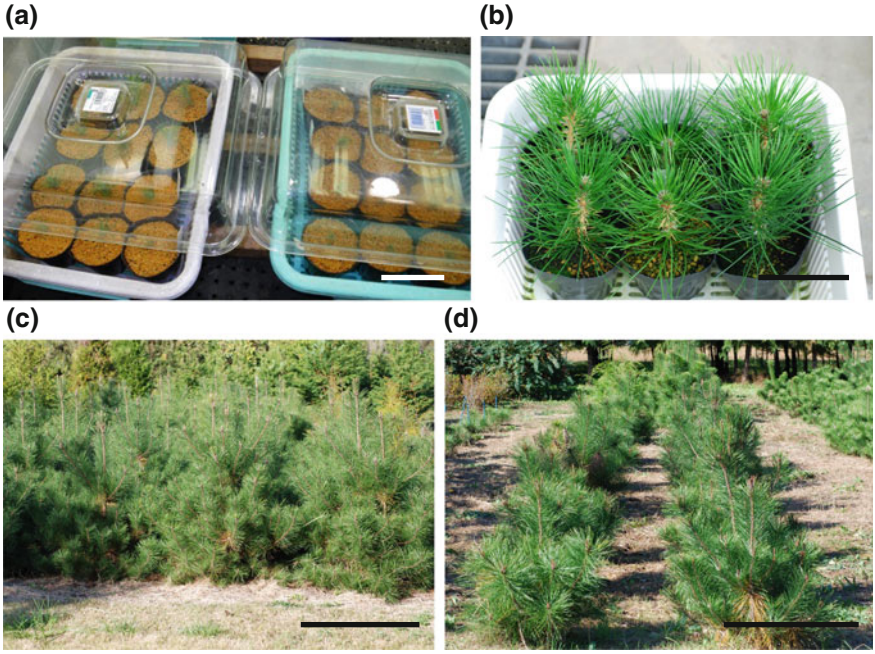

Fig. 8 Acclimatization and field transfer of somatic plants. **a** Acclimatization in plastic boxes, **b** acclimatized plants growing in a greenhouse before transplanting to the field, **c**, **d** somatic plants of Japanese black pine and Japanese red pine growing in the field. *Bars* **a**, **b** 10 cm, **c**, **d** 1 m

2. For the first 2 week somatic plants are kept under high relative humidity inside plastic boxes with transparent covers. During the first 2 weeks irrigate only with water as needed.
3. After the first 2 week, open the covers gradually and irrigate the pots with Hyponex nutrient solution as described in Table 2 (Fig. 8a).
4. Remove covers completely 4 wk after transplanting.
5. Best acclimatization and growth of somatic plants are recorded keeping the pots inside the growth chamber (80% relative humidity, and alternating temperature of 25 °C for 16-h photoperiod and 20 °C for 8-h darkness).
6. Subsequently, transfer the acclimatized plants to a greenhouse until they reach an approximate height of 15–20 cm (Fig. 8b) to be transplanting to the field preferably in spring season.
7. Remove somatic plants from pots and transplant to the permanent field location (Fig. 8c, d).

3 Conclusions and Future Prospects

An improved propagation system has been achieved for Japanese black pine and Japanese red pine with described protocol based on somatic embryogenesis initiated from immature seeds and plant regeneration obtained from somatic embryos after maturation on medium with polyethylene glycol. Post-maturation treatment based on the desiccation of somatic embryos after polyethylene glycol-mediated maturation markedly increased germination frequencies and synchronization during the germination period (Maruyama and Hosoi 2012b). However, further efforts are needed to establish an optimal protocol for the commercial production. Protocol modifications to increase the induction frequency of embryonal masses, as well as to develop efficient bioreactors that can be used for the large-scale production of somatic embryos are necessary. On the other hand, since most tree breeding programs have adopted a system of recurrent selection, strategies using vegetative propagation have additional advantages over traditionally improved seeds (Park 2002). Although at present, embryogenic systems derived from vegetative explants of mature pines have been reported in a few species (Texeira da Silva and Malabadi 2012), the positive results on somatic embryogenesis induction attributed to the reported methods have largely proven unrepeatable with other pine species (Trontin et al. 2016). For a more efficient implementation of somatic embryogenesis in tree breeding programs, more efforts are needed to develop a methodology to control the initiation of somatic embryogenesis from adult vegetative explants of Japanese pines.

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Cork Oak *Quercus suber* L. Embryogenic Liquid Cultures

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1 Introduction

The cork oak is a sclerophyllous evergreen tree species of the Fagaceae family. It is one of the most common hardwoods of the Mediterranean ecosystem. The native distribution of the species is in the coastal areas of the west-central Mediterranean Basin, including several African (Morocco, Algeria, Tunisia) and European (Portugal, Spain France, Italy) countries, as well as in their islands Majorca, Minorca, Corsica, Sardinia and Sicily (Gil and Varela 2008). There is a small presence of the species in the Adriatic sea coasts, in the regions of Istria and Dalmatia (Croatia), and Apulia, southeastern Italy (Schirone et al. 2015). The largest forests are in the Atlantic coast of the Iberian Peninsula and Morocco, country in which is located the 60,000 ha *Quercus suber* forest La Mamora (Ben Ali and Lamarti 2014).

The cork oak has great ecological and socio-economic importance. Both in Morocco and in the Iberian Peninsula the species is part of various agroforestry systems, which are called "montado" in Portugal and "dehesa" in Spain (Ben Ali and Lamarti 2014; Álvarez 2016). These system, besides cultural and conservation of biodiversity values, have a multifunctional use. Diverse agricultural, forestry and livestock production activities are performed in those areas, which are essential for the rural populations living in them. These agrosystems are threatened by several natural an anthropogenic perturbations that are leading to deforestation (Costa et al. 2014). Among them one of the most serious hazards is the oak decline called "la seca", mainly caused by a root infection by *Phytophthora cinammomi*.

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This syndrome is causing a high mortality in the last years (de Sampaio e Paiva Camilo-Alves et al. 2013).

The most characteristic production of *Quercus suber* is cork, although acorn crops are also important for feeding pigs of the Iberian race that are the basis of a high-quality food industry (Pugliese and Sirtori 2012). Recently, acorns of oak species are also attracting attention because of not only their food value but also for their applications in the cosmetic and pharmaceutical industries (Vinha et al. 2016). Cork is a renewable raw material, collected each 9–12 years from the trunk of cork oak trees. Its current main application is for stopping bottles of high-quality wine, but due to their diverse physical properties it is used for many applications in the construction, transport and aerospace industries (Duarte and Bordado 2015). In addition, by-products of the cork-processing industry can be useful due to their antioxidant (Aroso et al. 2017), biocide (Moiteiro et al. 2006) and growth-limiting of cancer cells (Bejarano et al. 2015) activities.

The products obtained from the species justify the accomplishment of genetic improvement programs. Current strategies of forest tree breeding emphasize on the use of vegetative propagation to rapidly capture all the potential of selected individuals and establish them in plantations, performing the so- called Multi-Varietal Forestry (Park et al. 2016). Somatic embryogenesis (SE) is one of the enabling technologies to carry out that strategy (Lelu-Walter et al. 2013). *Quercus suber* is the species within oaks in which SE has reached the most advanced development. The historic progress of SE in cork oak was widely described by Hernández et al. (2011). A review on plant regeneration of oak species by SE has been recently published (Corredoira et al. 2014).

We described the detailed step-wise protocol to clone adult cork oak trees in the former book of this series (Toribio et al. 2005). This protocol is robust enough to capture any genotype. In fact we cloned all the historic centenary *Quercus suber* trees of the Madrid Region (Spain) using this protocol (Ruiz-Galea et al. 2011). The protocol is adequate to produce the limited number of plants needed to establish clonal tests or for conservation purposes of selected trees. However for large-scale profitable clonal propagation, the scaling-up of the protocol using culture in liquid medium have to be developed.

The first description of somatic embryo formation in liquid medium was given by Puigderrajols et al. (1996), who cultured small cell clumps detached from proliferative masses produced in semisolid cultures of cork oak embryogenic lines. Further work on this issue has been reported, including the initiation of cultures in liquid medium, the maintenance of these cultures, the establishment of embryogenic suspensions, and the differentiation of single somatic embryos from these cultures, both in stationary and temporary immersion systems (Alegre et al. 2011; Jiménez et al. 2011, 2013; Jiménez 2013; Pérez et al. 2013). Herein we describe step-wise protocols to perform the production of somatic embryos obtained from selected *Quercus suber* trees using culture systems based on liquid medium.

2 Protocol of Embryogenic Liquid Cultures in Cork Oak

2.1 Materials

1. Plant material: embryogenic lines growing on semisolid proliferation medium.
2. High-purity water, mineral salts and organic components for preparing culture media; agar (S 1000, B&V, Parma, Italy); activated charcoal (ref. C9157, Sigma-Aldrich, Saint Louis, MO, USA).
3. Precision balance and autoclave. Inverted stereoscopic microscope.
4. Glassware: autoclavable 1000-mL wide-mouth bottles with screw caps; 1000-ml beakers.
5. Culture vessels: 150-ml baffled Erlenmeyer flasks; 250-ml Erlenmeyer flasks; Disposable 90-mm diameter plastic Petri dishes.
6. Sealants and caps: Parafilm® M (ref. P7793, Sigma-Aldrich, Saint Louis, MO, USA); Silicone sponge closures (Part #: 2004-00004, Bellco Glass, Inc., Vineland, NJ, USA).
7. Fractionation: Stainless steel sieve, 800-μm mesh; Hydrophilic Nylon Net Filter of 47 mm diameter with a 41 μm pore size (reference NY4104700, Merck-Millipore, Darmstadt, Germany). Büchner funnel, Kitasato flask and vacuum pump.
8. Filter-paper disks (80 g/m^2, 43–48 μm pore; Filter Lab, ANOIA, Barcelona, Spain).
9. Stainless steel spoons, tweezers. Glass bead sterilizer.
10. Horizontal orbital shakers (30 mm radius of movement).
11. Laminar-flow cabinets with ultraviolet light.
12. Tissue culture growth chambers.

2.2 Culture Medium

The basal culture medium for initiating liquid cultures, for their maintenance, for establishing embryogenic suspensions, and for differentiating embryos is the described by Hernández et al. (2003). It contains the macronutrients of Schenk and Hildebrandt's (1972), the micronutrients, cofactors and Fe-EDTA of Murashige and Skoog's (1962), and 30 g/L sucrose. For maturation of somatic embryos a culture medium composed of macronutrients of GD medium (Gresshoff and Doy 1972) and the rest of components of MS is also used. The pH is adjusted to 5.75 and the medium is autoclaved at 121 °C for 30 min. When gelled medium is required (embryo differentiation and maturation), 6 g/L agar is used (S1000, B&V, Parma, Italy).

2.3 Initiation of Embryogenic Liquid Cultures

Explants for initiating cork oak embryogenic liquid cultures are embryo clusters from proliferating cultures growing on a semisolid medium composed by the basal medium described above lacking plant growth regulators (PGR) (Fernández-Guijarro et al. 1995).

1. Pick up embryo clusters grown on semisolid medium and transfer them into 150-mL baffled Erlenmeyer flasks (7 g per flask) each containing 70 ml of liquid basal medium.
2. Shake vigorously the flasks by hand for a few seconds.
3. Remove the biggest clumps by sieving through a 800-μm steel sieve.
4. Collect the cells and cell aggregates released from embryo clusters after the brief shaking by pouring the liquid culture into a nylon mesh of pore size 41 μm.
5. Pick with a spatula the filtered materials and put them into 150-mL baffled Erlenmeyer flasks (all the amount of the fraction 41–800 μm released from the 7 g of embryo clusters per flask) filled with 70 ml of liquid basal medium without PGR, capped with silicone sponge closures.
6. Culture the flasks on horizontal orbital shakers (30-mm radius of movement) at 110 rpm placed in a growth chamber at 25 °C and a 16-h photoperiod (180 μmol m^{-2} s^{-1} photon flux density) for 4 weeks.

Embryo clusters of some genotypes picked from proliferating cultures on semi-solid medium hardly produce detachable materials after the brief shaking. When this happens, directly culture the embryo clusters as described in step 5 and follow the protocol. In these cases it is compulsory to remove the biggest and differentiated materials with tweezers after the first week of culture, and replace the culture medium if it becomes dark.

After the four weeks in liquid culture a mean of 16,300 structures per liter of culture medium can be obtained. These structures comprise single cells, small groups of cells with no defined structure, different sized and shaped embryogenic clumps (EMC) and cotyledonary embryos. The EMC are opaque white or translucent dense structures with a smooth surface. The smaller ones are mostly spherical and the larger ones may display spherical or oblong shapes, or complex nodular forms. The highest number of structures is within the fraction of 41–180 μm in size, being the number of structures of larger sizes (180–800 μm and >800 μm) smaller. The latter are capable of differentiating cotyledonary embryos when cultured on semisolid basal medium lacking PGR.

2.4 Maintenance of Embryogenic Liquid Cultures

When single EMC are cultured in liquid medium as inoculum for maintenance, they again produce new embryogenic materials. When only one EMC is cultured the produced materials show great variability among replicates both in the number of

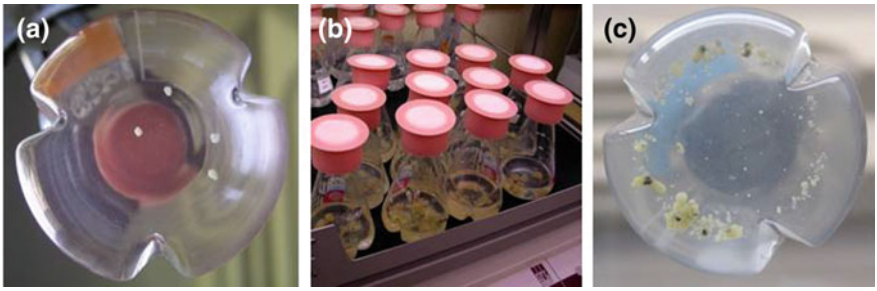

Fig. 1 Maintenance of embryogenic liquid cultures of cork oak. **a** Four spherical EMC of 1–2 mm diameter, **b** flasks on horizontal orbital shakers (30-mm radius of movement) at 110 rpm, **c** materials produced after four weeks in culture

structures and their size distributions, which are independent of EMC size, shape, or if they have presence or absence of secondary embryogenesis. Instead, when four EMC are cultured the variability is largely reduced.

1. Within the materials produced after the previous initiation protocol, collect spherical EMC of 1–2 mm diameter using tweezers.
2. Put them into 150-mL baffled Erlenmeyer flasks (4 EMC per flask) filled with 70 ml of liquid basal medium without PGR, capped with silicone sponge closures (Fig. 1a).
3. Place the flasks on horizontal orbital shakers (30-mm radius of movement) at 110 rpm placed in a growth chamber at 25 °C and a 16-h photoperiod (180 μmol m^{-2} s^{-1} photon flux density) for 4 weeks (Fig. 1b).
4. From the produced materials (Fig. 1c), collect again with tweezers new EMC as described in step 1 and follow the same protocol to start a new cycle of maintenance.

For the maintenance of embryogenic liquid cultures initial low density inoculums is used, around 43 mg/L. After 4 weeks in culture the culture density reaches a mean of 7.6 g/L of very heterogeneous materials. From them many EMC can be collected to begin the maintenance cycle again, and to be able to maintain the cork oak liquid cultures for years without losing the embryogenic ability.

2.5 Establishment and Maintenance of Embryogenic Suspensions

Obtaining true suspension cultures is not easy with embryogenic cork oak tissues. True suspension are those composed of homogeneous structures, typically single cells, small cell aggregates without structuring, or globular embryos growing at high densities. They are usually achieved in other species using PGR, mainly

auxins. These cannot be done successfully in cork oak. However, true suspension can be got by managing the physical parameters of cultures, such as type and size of vessel, volume of culture medium, orbiting speed, type and size of initial inoculums, and mostly density of inoculation.

1. To begin this protocol use the materials produced at the end of step 3 of the maintenance protocol. Depending on genotype, the amount needed to inoculate one flask for establishing suspension cultures is produced in 1–5 flasks.
2. Remove the biggest clumps by sieving through a 800-μm steel sieve, and then collect the fraction 41–800 μm by pouring the liquid culture onto a nylon mesh of pore size 41 μm. To do that, put the nylon mesh in a funnel placed in a kitasato flask connected to a vacuum pump, and apply low suction pressure while filtering.
3. Take the filtered materials with a spoon and put them into 250-mL Erlenmeyer flasks (400 mg per flask) filled with 50 ml of liquid basal medium without PGR, capped with silicone sponge closures (Fig. 2a).
4. Incubate the flasks on horizontal orbital shakers (30-mm radius of movement) at 110 rpm placed in a growth chamber at 25 °C and a 16-h photoperiod (180 μmol m^{-2} s^{-1} photon flux density) for 4 weeks.

After culturing the 41–800-μm fraction at initial high density of inoculation (8 g/L) for one month, true suspension cultures are obtained (Fig. 2b). They comprise very homogeneous structures with a high frequency of the ones of globular type (Fig. 2c, d). The biomass produced at the end of this period is between 5 and 13 g per flask, depending on genotype, from which the fraction <800 μm is around 30%. Suspension cultures can be maintained by repeating the same protocol starting in the step 2.

2.6 *Differentiation of Somatic Embryos*

The goal of the scaling-up in the production of somatic seedlings is to obtain single embryos without secondary embryogenesis able to germinate. Differentiation of somatic embryos in liquid cultures may follow three pathways. In the first one, embryos develop from embryogenic tissues (both previously differentiated embryos, proliferating masses and EMC) following the pattern usually observed in cultures on semisolid medium. Differentiation begins with the formation of shoot apex and cotyledons, and the new embryos outgrowth from the initial tissues (Fig. 3a) forming clusters of somatic embryos attached at their root poles. Few embryos detach from these clusters, and consequently the production of single embryos is low.

The second way of differentiation is also observed in differentiated embryos and in organized structures; in this case the new embryos are formed as structures that looks like globular embryos loosing from their surface (Fig. 3b). Both pathways

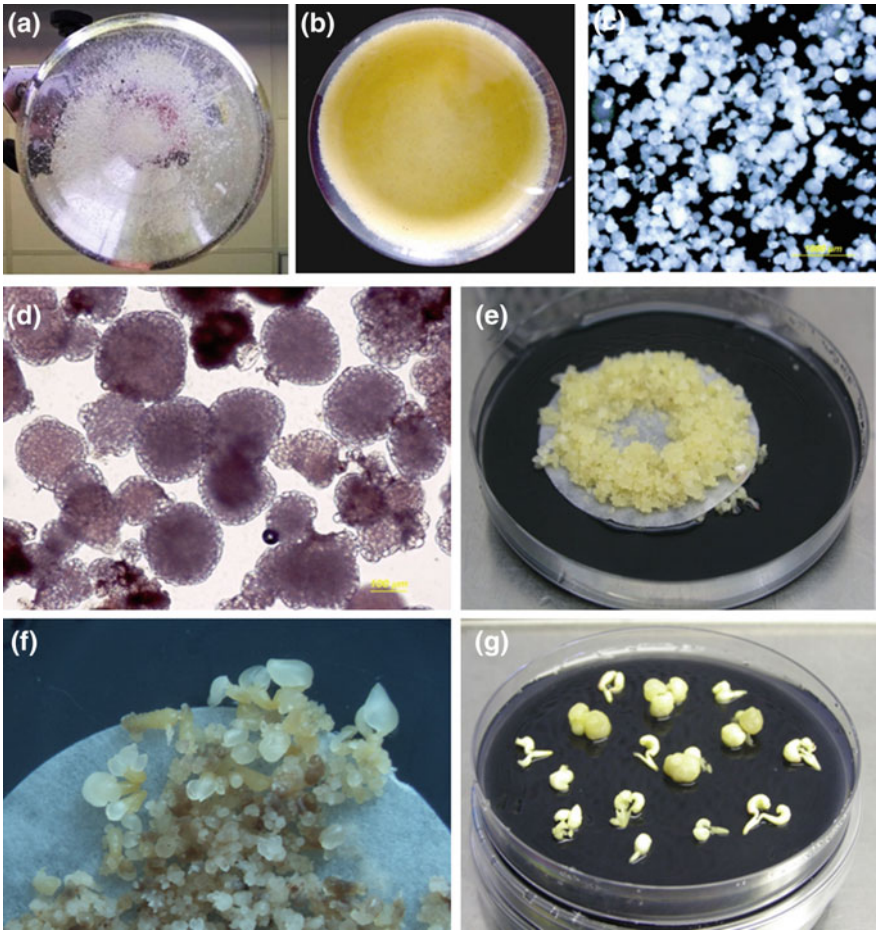

Fig. 2 Production of somatic embryos from suspension culture, differentiation and maturation. **a** 41–800-µm fraction at initial high density of inoculation (8 g/L), **b** suspension produced after 4 weeks, **c** microscopic image of a true suspension culture, **d** detail of globular structures, **e** differentiation of embryos from suspension culture on paper-filter disk, **f** immature embryos, **g** mature embryos

were described by Puigderrajols et al. (1996) as having multicellular (the first one) and unicellular origin (the second one). In the third way, small non-organized aggregates of few embryogenic cells in suspension cultures are able to develop globular embryos (Fig. 3b). The two later pathways produce single embryos that develop free in the liquid medium.

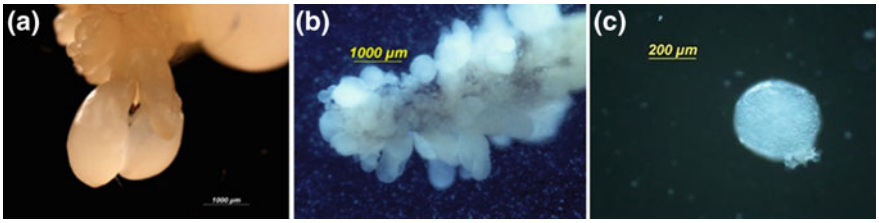

Fig. 3 Three ways of differentiation of somatic embryos observed in liquid cultures of cork oak, **a** embryos developing from embryogenic tissues, **b** embryos formed as structures that looks like globular embryos loosing from embryos or differentiated structures, **c** globular embryo developed from small non-organized aggregates of embryogenic cells in suspension cultures

2.6.1 Differentiation from Embryogenic Structures Growing in Liquid Medium

Differentiated structures cultured at low density of inoculation and low speed or stationary condition produce globular structures on their surfaces that develop into free and well-formed cotyledonary embryos (Fig. 4a).

1. Pick immature somatic embryos or polar structures (>800 µm) from maintenance cultures at the end of step 3 and culture them in 150-mL Erlenmeyer flasks (2 embryos or structures per flask) with 50 mL of basal medium lacking PGR, capped with aluminum foil.
2. Place the flasks stationary in a growth chamber at 25 °C in darkness for 60 days.

After that period of time a mean of 23 single embryos per flask can be obtained (Fig. 4b, c), but this production ability is highly dependent on genotype. Higher densities of inoculation or smaller flasks reduce the number of produced embryos. If these embryos are again used to produce new embryos using the same protocol, production decreases as more cycles of production are carried out.

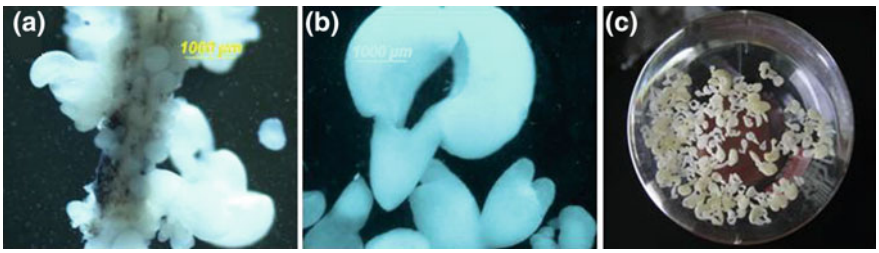

Fig. 4 Production of somatic embryos from differentiated embryogenic structures growing in liquid medium, **a** globular structures arose and develop on the surface, **b** free and well-formed cotyledonary embryos, **c** flasks with embryos produced at the end of the protocol of differentiation

2.6.2 Differentiation from Suspensions

Cork oak cells and cell aggregates growing in true suspensions can originate single somatic embryos. Current protocols have been developed using semisolid medium.

1. Collect the fraction 41–800 μm from maintained suspension cultures at the end of step 4 following the same procedure described in step 2.
2. Disperse 50 mg of materials of this fraction in a small volume of liquid basal medium. Pour it onto a filter-paper disk placed in a funnel joined to a kitasato flask connected to a vacuum pump, and apply low suction pressure for removing the liquid medium.
3. Lay the filter paper with the embryogenic materials on the medium surface in a 90-mm diameter Petri dish filled with 20 mL of basal medium, supplemented with 5 g/L active charcoal and gelled with 6 g/L agar.
4. Place the Petri dishes sealed with Parafilm® in a growth chamber at 25 °C in darkness for 30 days.

At the end of this time period around 500 single embryos per gram of embryogenic tissue can be picked-up for maturation. There is also a great production of new embryogenic tissues (Fig. 2e, f). The production of single embryos is highly influenced by the presence of active charcoal, but not by the macronutrient composition of basal medium (SH versus GD) nor by the addition of 15 or 50 μM ABA.

2.7 Maturation of Somatic Embryos and Plant Recovery

The same protocols described in the first book of this series (Toribio et al. 2005) can be used to mature embryos obtained from liquid cultures and suspensions, and to convert into somatic seedlings. Alternatively, some modifications are available for maturation of somatic embryos.

1. Pick immature embryos of 4–10 mm in length formed in stationary liquid cultures or on the surface of the filter-paper disks.
2. Put them in 90-mm Petri dishes filled with 20 mL of culture medium with GD macronutrients supplemented with 5 g/L active charcoal and gelled with 6 g/L agar.
3. Place the Petri dishes sealed with Parafilm® in a growth chamber at 25 °C in darkness for 30 days.
4. Move the Petri dishes into a refrigerator at 4 °C in darkness, and leave them there for 2 months.

At the end of step 3 the weight of somatic embryos is tripled, from 90 to 280 mg mean fresh weight. After the two months under cold treatment the mean fresh weight reaches 910 mg. Some embryos are lost because they begin a new cycle of secondary embryogenesis, between 15 and 28% depending on genotype. After this period the embryos are ready for germination (Fig. 2d).

3 Research Prospects

The initiation of cultures in liquid medium and especially the establishment of suspensions is difficult in some species like the cork oak, in which the secondary embryogenesis occurs in medium without PGR and at a very advanced state of development of the embryos (Merkle et al. 1995; Jiménez et al. 2013). At present, the described protocols are developed enough to initiate cork oak embryogenic cultures in liquid medium, to maintain these cultures, and to establish true embryogenic suspensions. Further research is needed to refine methods to differentiate somatic embryos at large scale.

Current protocols for suspension cultures allow the production of high amount of biomass in short time. This system offer great promise for the production in bioreactors of the secondary metabolites that have been identified in cork oak kernels as having cosmetic and medicinal properties. Particularly important are those that have the potential for treating diabetes and neurological disorders (Custódio et al. 2015).

Although the production of single somatic embryos ready for germination is relatively high with the current protocols, its improvement is still a challenge. Some preliminary experiments indicated that the use of semi-permeable membranes placed on semisolid medium (Niedz 2006) may be useful for this purpose. However the production of embryos on semisolid medium is a labor demanding procedure. Therefore the goal should be the differentiation of single somatic embryos in liquid medium, followed by their maturation and even pregermination in temporary immersion systems, as is done with other species such as coffee (Ducos et al. 2007; Etienne et al. 2013). Preliminary experiments showed that single cork oak somatic embryos can be differentiated from suspensions in liquid medium by lowering the culture density and maintaining the cultures under stationary condition.

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Norway Spruce *Picea abies* (L.) Karst

Saila Varis

1 Introduction

The increasing use of wood as a source of bioenergy, bio-products and conservation of more natural (old) forests with high biodiversity, compel us to find means to increase forest productivity. Using the best quality regeneration material can increase the economic gain obtained from future silvicultured forests. Norway spruce is an important raw material in the European forest industry and it is the most-planted tree species in Finland. However, there is periodically a lack of high-quality Norway spruce seed due to irregular flowering of the species, as well as pests and pathogens which can lower the productivity of seed orchards. To ensure availability of good-quality forest regeneration material, effective vegetative propagation methods like somatic embryogenesis (SE) can be introduced. SE has become the method of choice for vegetative propagation of conifers (Sutton 2002) due to its high multiplication rate and the maintenance of juvenility via cryopreservation that allows long-term field testing of materials.

Research of SE methods in Norway spruce started over 30 years ago (Chalupa 1985; Hakman and von Arnold 1985; Jain et al. 1988) and since then protocols have been developed in different laboratories (Vagner et al. 2005; Högberg and Varis 2016). In vitro regeneration is dependent on genotype, physiological state of tissue, and culture conditions. For example the frequency of successful initiations of Norway spruce zygotic embryos varies between crossings (Högberg and Varis 2016), immature zygotic embryos grow embryogenic tissue more often than mature, and half-strength basal medium enhanced embryogenic callus formation in Norway spruce mature zygotic embryos compared with full-strength medium (Jain et al. 1988). However, conditions and handling throughout the cultivation process

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affect the final result of SE production, many critical phases can be identified from the production process and all of them cumulatively add to the production costs of SE-plants if they cannot be controlled. Somatic embryo development from proembryogenic masses through maturation phases to mature embryo is well described (Filonova et al. 2000), and in recent studies attention has been paid for genetic regulation of the SE development (von Arnold et al. 2016). Research effort has also been put to automatization of the Norway spruce SE production, field testing, and integration of the SE production to breeding programs using emblings as donors for the field test material (Högberg and Varis 2016; Tikkinen et al. 2017).

In this chapter some critical phases are pointed out and improved protocol for Norway spruce SE plant production is described.

2 Protocol of Somatic Embryogenesis in Norway Spruce

2.1 Culture Medium

The most commonly used media for culture of Norway spruce embryogenic tissue (ET) are based on either MS (Murashige and Skoog 1962) or LM (Litvay et al. 1985), which different laboratories have more or less modified (von Arnold and Eriksson 1981; Gupta and Durzan 1986; Jain et al. 1988; Klimaszewska et al. 2001). Here the modified LM (mLM) medium (Klimaszewska et al. 2001) is presented with some modifications based on recent research. The basal composition of mLM medium is listed in Table 1 and compounds whose concentrations vary between proliferation, maturation or germination media are listed in Table 2. Easy-to-use stock solutions can be made from combinations of macrosalts, microsalts and vitamins, or they can be prepared in smaller lots to help traceability in problem situations (Tables 3 and 4). For the same reason stock and media lots are identified with a running number (Table 5). Stocks can be stored in −20 °C up to six months, and thawed stocks in +4 °C for two weeks.

The same mLM medium containing 2, 4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyladenine (BA) as plant growth regulators is applicable both for the induction and proliferation of embryogenic cultures of Norway spruce. The medium is solidified with gellan gum (Phytagel) but can be used in liquid form, for example, in temporary immersion system (TIS) bioreactors. Usually 90 mm petri dishes are used in cultivation in semi-solid medium, especially when automated petri dish filler is utilized, but also cheaper material like freezer boxes or reusable Magenta vessels are able to be used. Automatization helps when numerous petri dishes have to be filled.

For development of somatic embryos 2, 4-D and BA are replaced by (+)-abscisic acid (ABA). The amount of ABA varies in different recipes from 20 to 60 μM (Högberg et al. 2001; Klimaszewska et al. 2001; Vondráková et al. 2014) being 60 μM in original mLM. Because ABA also acts as an inhibitor of plant growth, the

Table 1 Basic compounds of modified LM medium (Klimaszewska et al. 2001) for Norway Spruce based on Litvay et al. (1985)

Compound	mg/l
NH_4NO_3	825
KNO_3	950
$MgSO_4 \times 7H_2O$	925
KH_2PO_4	340
$CaCl_2 \times 2H_2O$	22
H_3BO_3	31
$MnSO_4 \times 2H_2O$	21
$ZnSO_4 \times 7H_2O$	43
$Na_2MoO_4 \times 2H_2O$	1.25
$CuSO_4 \times 5H_2O$	0.50
$CoCl_2 \times 6H_2O$	0.125
KJ	4.15
$FeSO_4 \times 7H_2O$	27.80
$Na_2EDTA \times 2H_2O$	37.30
Myo-inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.1
Thiamine HCl	0.1
Casein hydrolysate	1000
L-glutamine	500

Table 2 Variable compounds in mLM medium for Norway Spruce

Compound	Initiation and proliferation	Maturation	Germination
NH_4NO_3 (mg/l)	825	825	
Sucrose (g/l)	10	60	20
Phytagel (g/l)	4	6	6
2,4-D	10 μM		
BA	5 μM		
ABA		30 μM	

high concentration or long contact with it may negatively affect germination and further growth of Norway spruce somatic embryos (Högberg et al. 2001). On the other hand, using a low concentration may require moving of embryos to fresh media during their development (Vondráková et al. 2014). In our experiments embryos developed in a 30 μM ABA concentration without moving to fresh media, and mature embryos had higher germination rates than embryos maturated in a 60 μM ABA concentration (Tikkinen et al. 2018a). High sucrose concentration, and in some cases polyethylene glycol 4000 (PEG), are common in maturation media. Both substances induce osmotic pressure which results in an increased amount of

Table 3 Example of the compositions of stocks for mLM medium

Stock	Ingredients	g/l
LM-NO_3	KNO_3	95
LM-NO_3	NH_4NO_3	82.5
LM-SO_4	$MnSO_4 \times H_2O$	2.1
LM-SO_4	$ZnSO_4 \times 7H_2O$	4.3
LM-SO_4	$CuSO_4 \times 5H_2O$	0.05
LM-J + Cl_2− ½− $CaCl_2$	KJ	0.415
LM-J + Cl_2− ½− $CaCl_2$	$CoCl_2 \times 6H_2O$	0.0125
LM-J + Cl_2− ½− $CaCl_2$	$CaCl_2 \times 2H_2O$	1.1
LM-P + B + Mo− ½KH_2PO_4	KH_2PO_4	17
LM-P + B + Mo− ½KH_2PO_4	H_3BO_3	3.1
LM-P + B + Mo− ½KH_2PO_4	$Na_2MoO_4 \times 2H_2O$	0.125
LM-Vitamins	Tiamiini HCl (B_1)	0.01
LM-Vitamins	Nikotiinihappo	0.05
LM-Vitamins	Pyridoksiini (B_6)	0.01
LM-Vitamins	Tiamiini HCl (B_1)	0.01
Myo-inositol	Myo-inositol	40
NaFe-EDTA	NaFe-EDTA	4

Table 4 Hormone stocks for mLM medium

Stock	Solvent	mg/10 ml
2,4-D	EtOH/1 N NaOH	221.0
BA	1 N NaOH	225.3
ABA	1 N NaOH	264.3

Dilute in small amount of EtOH or NaOH and adjust volume with water

mature embryos (Tremblay and Tremblay 1995). However, PEG has had negative effects on embryo germination and root growth (Bozhkov and von Arnold 1998) and is therefore not included in the Norway spruce medium presented here. For maturation in TIS bioreactors, the media presented here requires further optimization (Salonen et al. 2017).

Mature embryos are germinated on medium without any plant regulators. The chemical form of nitrogen influences root:shoot ratio in growing seedlings of Norway spruce in a way that the organic nitrogen source seems to enhance root growth when compared to seedlings cultivated on an inorganic nitrogen source (Gruffman et al. 2012). Increasing the ratio of organic nitrogen by not including NH_4NO_3 in the mLM germination medium resulted in increased root length and higher root: shoot ratio (Tikkinen et al. 2018b *in press*).

Table 5 Example of the "Cook Book Sheet" for mLM proliferation medium

mLM proliferation medium, ½ macroelements			
Lot number	Stock	Amount/l	Amount/medium
–	LM-NO_3 stock	10 ml	–
–	LM-SO_4 stock	10 ml	–
–	LM-J + Cl_2− ½− $CaCl_2$ stock	10 ml	–
–	LM-P + B + Mo− ½KH_2PO_4 stock	10 ml	–
–	LM-Vitamins stock	10 ml	–
–	Myo-inositol stock	2.5 ml	–
–	NaFe-EDTA stock	10 ml	–
	$MgSO_4 \times 7\ H_2O$	0.925 g	–
	Casein hydrolysate (N-Z-Amine®A)	1 g	–
–	2,4-D stock	100 µl	–
–	BA stock	50 µl	–
	Sucrose	10 g	–
	pH	5.8	–
	Ad. maxim water	1000 ml	–
	Phytagel	4 g	–
Add after autoclaving:			
	L-glutamine (0,2 µm filter)	500 mg	–
	Solubility 1 g/40 ml		

Prepare medium without L-glutamine. Take appr. 20 ml of medium, add L-glutamine into it and use 20 ml syringe with 0.2 um filter to add it into autoclaved medium after cooling it to 60 °C. Dosing 20–25 ml of medium per petridish

2.2 Explant Preparation and Proliferation of Embryogenic Culture

Both mature and immature zygotic embryos can be used as explants in the initiation of Norway spruce embryogenic cultures, although the initiation frequencies are lower with mature embryos compared to immature ones. Following the methodology developed for the induction of ET from primordial shoots of mature trees, successfully applied in White spruce (Klimaszewska et al. 2011), induction of SE and regeneration of emblings has recently been achieved also in Norway spruce (Varis et al. 2018 *in preparation*). However, the frequency of initiation from Norway spruce shoots has been low.

Immature cones are collected in summer when the heat sum is around 800 degree days and they can be stored in +4 °C for one week (Varis et al. 2018, *in press*). Cones are cleaned with 70% ethanol, seeds are dissected and placed in sterile water and a drop of dishwashing soap is added to clean the seeds. After one or two rinse cycles in sterile water, seeds are surface sterilized in 70% ethanol for 5 min and rinsed three times in sterilized water. Washing and rinsing can be done either in a petri dish or with the help of a tea ball. Megagametophytes are carefully

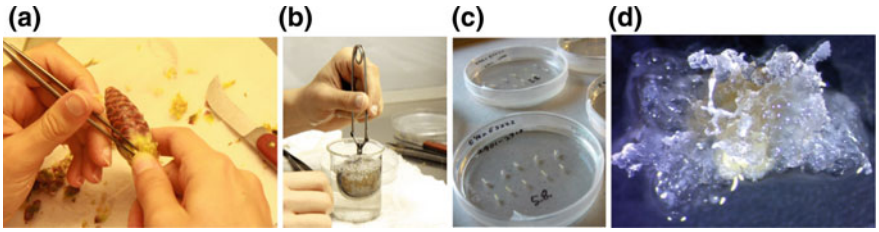

Fig. 1 Initiation and proliferation of embryogenic tissue from Norway spruce immature zygotic embryos. **a** Immature seeds are dissected from the cone using knife for loosening scales and forceps for taking seed carefully out; **b** tea ball is handful when seeds are surface-sterilized in ethanol and washed in sterilized water; **c** zygotic embryos dissected from seed are placed on medium. Crossing, embryo numbers and initiation date are market on the lid; **d** embryogenic tissue grows in 3–8 weeks

opened with a scalpel under a stereo microscope and zygotic embryos are placed on the medium. Usually ten zygotic embryos are placed on each Petri dish and each embryo is systematically labeled which may be part of or all of the future line number. Embryos are kept in the dark at 24 °C on the same medium until embryogenic tissue (ET) has grown 5–10 mm and is ready to be excised from the zygotic embryo to establish an embryogenic line (Fig. 1).

Established ETs are subcultured at 12–14-day intervals. ET can be proliferated on semi-solid media in clumps, or spread on the filter like in the maturation phase (see below). Using suspension cultures in continuous rotation seems to be too rough a treatment for most of the cell lines. As an alternative to suspension cultures, Norway spruce ET proliferates well when it is only temporarily immersed into liquid media.

Good looking Norway spruce ET is bright white, with brown spots sometimes forming quite soon after subculturing. Typically the brown parts are avoided, as the newest bright tissue is preferred in subculturing. However, brown spots or brownish coloring in the old growth has no effect on embryo production or viability of cryopreserved ET when the tissue used in maturation and cryopreservation is the newest growth. Maturating and cryopreserving of the Norway spruce tissue should be done as soon after initiation as possible using the newest tissue from cultures to ensure the best embryo production and recovery (Varis et al. 2018, *in press*).

The protocol of initiation from primordial shoots is presented here according to the findings of Klimaszewska et al. (2011) and applied for Norway spruce. Shoot buds can be collected either in the spring before the growing season or after it in the autumn. Lateral buds can be stored in 2–4 °C for up to 5 days. The basal scales of buds are removed, and buds of each genotype are divided into two 50 ml plastic tubes. The outer resin is washed away by adding 94% ethanol and shaking the tubes for one minute. Washing and disinfecting continues with six minutes of shaking in tap water including a small amount of Tween-20, two minutes of shaking in 70% ethanol, and eight minutes of shaking in 10% (v/v) hydrogen peroxide. After rinsing three times in sterile water, buds are placed on a Petri dish with 100 mg l^{-1}

polyvinylpyrrolidone (PVP) solution. For dissection a smaller amount of buds is placed in a new Petri dish on a filter paper soaked with PVP solution. Buds are cut in two lengthwise, primordial shoots are excised and depending on the size they are recut into two or three parts. Sections of the primordial shoots are placed with their cut surface towards the semi-solid mLM proliferation media. Bud explants are kept in the dark at 24 °C on the same medium and treated like zygotic embryos.

2.3 Maturation and Germination of Somatic Embryos

For induction of somatic embryo maturation about 180 (±20) mg of ET, five to seven days from the last subculture, is mixed in 3 ml liquid media without plant growth regulators (PGR). The suspension is poured onto a paper filter (Whatman #2) which is placed in a Büchner funnel. The liquid is drained by suction and the filter is placed on mLM maturation medium. Developing embryos are kept in the dark at 24 °C on the same medium for eight weeks.

Mature somatic embryos of Norway spruce can be partially desiccated to lower endogenous ABA levels and thus prevent after effects in embryo germination. Shortly: mature embryos are placed on filter paper in a petri dish which is placed on a larger vessel together with a petri dish containing sterile water. The whole package is sealed with parafilm and incubated in the dark for 16–24 days (Bozhkov and von Arnold 1998; Högberg et al. 2001). This procedure has increased germination frequency, but the same effect is also reached when mature embryos are cold stored on maturation media for one to six months (Varis et al. 2018, *in press*). Embryos should be stored at a stable temperature of 2–4 °C to avoid formation of condensation water inside the petri dish.

Mature Norway spruce embryos should be shortly germinated in vitro on PGR-free medium before acclimatization to greenhouse conditions (Fig. 2). It seems that the traditional five-week germination period (Klimaszewska et al. 2001) does not yield the best result regarding the later life of the embryo. For example, in recent studies (Tikkinen et al. 2018b *in press*) showed that the survival rate of

Fig. 2 Germination and acclimatization of Norway spruce somatic embryos **a** good mature embryos (left side) have well developed apical and root meristems, and at least four cotyledons; **b** germinated embryos after two weeks on hormone free media; **c** growing emblings in propagators placed under LED light system

Norway spruce SE emblings after the first growing season was higher when embryos were germinated only one week compared to five or three weeks germination. In addition to more vital plants, increased height growth was achieved with a shorter germination treatment.

Mature embryos are placed on petri dishes, ten to fifteen embryos per dish, in a way that the cotyledons face up when the petri dishes are placed in a slightly tilted position. Instead of germination on semi-solid media, embryos could be put on a metal mesh placed over liquid media which allows root development in liquid (Högberg and Varis 2016).

The light intensity can be low (5 µmol/m−2/s−1) at the beginning of the germination period and increase towards the end to 130–150 µmol/m−2/s−1. Intensity should be high enough, but the heating effect of the lightning system should be taken into account. The temperature in the germination room should be adjusted so that the target temperature for the germinating embryos inside the petri dishes or other vessels stays within a tolerable range. Attention has to be paid also to the spectrum of the lightning system; fluorescent lamps usually emit much more white wavelengths favorable to the human eye than the red and blue wavelengths that plants use. The photoperiod of the 18 h day/6 h night should be introduced at least for northern genotypes, while with southern genotypes 14–16 h day/10–8 h night may be enough.

2.4 *Acclimatization and Field Transfer*

Acclimatization of germinated emblings from in vitro to ex vitro conditions is usually the most stressful stage in the emblings' lives, no matter how careful you are and how optimized conditions are. Acclimatization can be done in a special greenhouse, funnels, or in commercial—either reusable or disposable—propagators, in which temperature and relative humidity can be controlled (Fig. 3). Propagators can be handy when sustaining high humidity is not possible or growing space is limited.

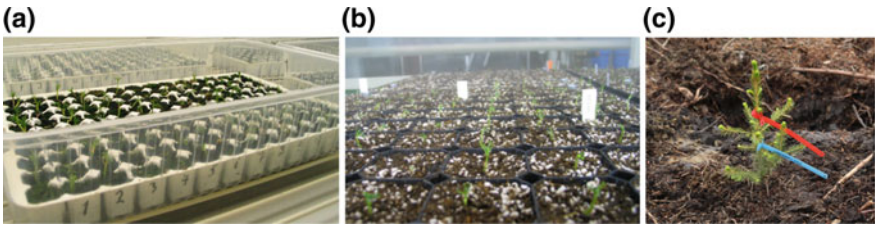

Fig. 3 Norway spruce emblings in the acclimatization and field **a** plastic lids have been removed when emblings have been acclimatized three weeks in propagators; **b** acclimatization in the funnel; **c** embling planted in the field to the spot which has been mounded

Attention has to be paid during every step when handling embryos, starting from the transferring procedure when drying must be avoided by keeping embryos out of media or peat for as short a time as possible and avoiding heating lamps. The temperature during acclimatization should be close to the germination temperature. The relative humidity should be high (85–90%) at the beginning of the growth ex vitro. After a critical period of two to three weeks it should be gradually decreased to the same level used in the greenhouse where seedlings are growing. The light intensity and day length should be at least the same as during germination to avoid the formation of an apical bud.

Growth media is often peat mixed with perlite or vermiculite or sand to increase water and nutrient retention and to aerate the peat. Also, commercially available mini-plugs can be used, especially in propagators. Watering and fertilizing should be done by carefully optimizing the concentration and amount of the liquid. Drying and the need for fertilizer liquid can be evaluated with the help of scaling.

For transferring germinated embryos to the peat the "pricking out" method (Landis et al. 2010) can be introduced: forceps and a modified screwdriver are used for making a small hole in the peat, transferring emblings and straightening the roots. After transferring, the peat should be gently compressed around the emblings to ascertain sufficient soil contact for the developing root (Tikkinen et al. 2018b *in press*).

2.5 *Preservation of Culture and Embryos*

Under continuous in vitro culture, embryogenic tissues may decline or even lose their somatic embryo differentiation ability. For maintenance of somatic embryo production capacity of selected lines, as well as for storage of these lines for future use, a reliable cryopreservation method is needed. A cryopreservation protocol based on cryoprotectants added in a suspension culture of Norway spruce and freezing with slow-cooling (Norgaard et al. 1993) is used in many laboratories. More recently, a cryopreservation protocol based on drying of embryogenic tissue of one cell line and direct immersion in liquid nitrogen (LN) without the use of cryoprotectants have also been described (Hazubska-Przybyl et al. 2013).

In recent studies by Varis et al. (2018, *in press*) different sets of classical pre-treatment with cryoprotectants and cooling methods were tested using a large set of genotypic material. The physiological condition of the tissues, pre-treatment and cryoprotectant applied, as well as the slow-cooling device used were found to affect the recovery of Norway spruce ET. The most reliable option was to select fresh growth from young ETs as samples, and pretreat them on semi-solid medium with increasing sucrose concentration (0.1 M for 24 h; 0.2 M for another 24 h). About 200 mg ET was placed in sterile cryovials (six to eight clumps in each 2 ml cryovial) containing 400 µl liquid mLM medium with 0.4 M sucrose concentration but without plant growth regulators, gelling agent or glutamine. Cryovials were placed on thermoconductive racks which were precooled in −20 °C. Into each

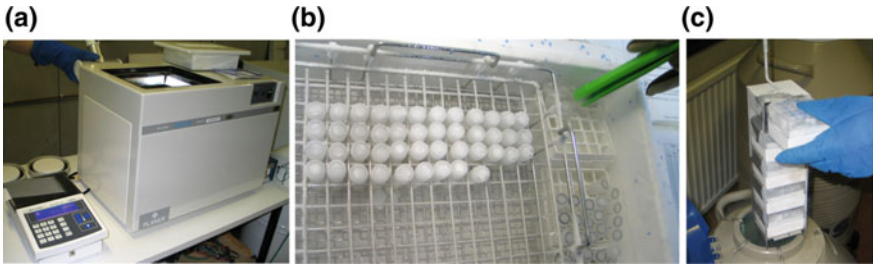

Fig. 4 Cryopreservation of pretreated samples. **a** Programmable freezer for slow cooling; **b** in the freezer cryovials are in the metal rack and should be moved to cryoboxes quickly, e.g. by placing them in styrox box with liquid nitrogen at the bottom of it; **c** cryoboxes are moved to liquid nitrogen containers as soon as possible

cryovial 400 µl pre-chilled cryoprotectant PGD solution (composed of polyethylene glycol 6000, glucose, and Me_2SO 10% w/v each) was added in 200 µl aliquots within the time period of 30 min. Cryovials were then incubated for 30 min in thermoconductive racks. Freezing of the samples should be done with the slow-cooling method using a programmable cooling device at the rate of 0.17 °C/min to −38 °C (Fig. 4).

When samples are taken from LN they should be immediately thawed in a water bath +37 °C for 2 min. Cryovials should be wiped with 70% ethanol and the content of the tube is poured onto the sterilized paper filter (Whatman #2) and placed in the Büchner funnel. The cryostorage liquid is drained off by suction and the tissue is washed with the same liquid mLM medium which was used in the pretreatment process. Samples are placed on medium with a sucrose content of 0.2 M, and transferred every 24 h on medium with decreasing sucrose concentration (0.1 and 0.03 M). All filters with tissue are transferred to new media every two weeks. With this method, on average 87% of the genotypes were recovered without any effect on their genetic fidelity, as shown by microsatellite markers and embryo production capacity (Varis et al. 2018, *in press*).

In Nordic tree species transfer of somatic plants from the laboratory to nursery for further growth is not possible during Northern winter conditions. Plant production using heated greenhouses with additional lights is possible, but not cost-efficient. Thus, storing mature embryos in cold enables production of somatic embryos in the laboratory throughout the year, and allows synchronization of their development and then germination for further cultivation at the proper time, i.e. spring. Even circa one-year on storage, using the method described earlier, resulted in higher germination percentages compared to fresh ones, however, part of the embryos had started to germinate during storage, and the amount of good-looking embryos was reduced (Varis et al. 2018, *in press*).

3 Research Prospects

For practical application of Norway spruce SE the most important aspect is to improve the cost efficiency of the whole process. Currently the SE-process described here and used in many laboratories is based on manual labor, which is time consuming and expensive. Automatization of the SE-process has been the focus of the research in some laboratories or companies for some time; developments include the application of bioreactors, mainly Temporary Immersion Systems (TIS), automated embryo selection and artificial seed production.

There are several commercially available TIS models in which liquid medium is applied at intervals to plant material that is located in a compartment that is separate from the medium. The advantages of the TIS systems in embryo germination and plant production have been documented with several commercially important species like coffee, and benefits have been shown to include the reduction in workload and thus in cost of the produced plant (Etienne and Berthouly 2002). However, only limited data are available on the effect of the different culture parameters, such as the immersion frequencies, on ET culture during the proliferation and maturation stages. In Norway spruce SE, the proliferation and maturation of ETs in TIS bioreactors has been achieved, although especially the maturation phase could still be further optimized to increase propagation efficiency (Salonen et al. 2017). Optimizing may be challenging and time consuming, with all the culture process steps having to be optimized for a wide variety of genetic materials. Besides productivity of the cultures, the cost-efficiency and easiness of sterilization and aseptic handling of TIS during the culturing process is important. TIS models commercially available at the moment are designed for growing plants, thus they are higher than needed for tissue growth and one vessel takes too much space in the autoclave.

Automated embryo selection and planting of the germinated embryos into peat could remarkably reduce the manual work. Many kinds of modern seed planting machines are available, but applications for somatic embryos are rare.

Despite recent breakthroughs (Klimaszewska et al. 2011) the problem of tissues of mature conifers being generally recalcitrant for SE induction requires further research. The possibility to propagate mature conifer trees with known characters via SE in combination with cost-efficient mass propagation would have an enormous impact on forestry with subsequently increased productivity and/or production of tailored raw materials for special end-users.

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Eucalypts (*Eucalyptus globulus* Labill.)

Elena Corredoira, Ana M. Vieitez and Antonio Ballester

1 Introduction

The most widely planted hardwood in the tropical and subtropical world is *Eucalyptus*, a fast growing evergreen tree of the *Myrtaceae* family. Several species of the 894 taxa that belong to the genus, such as *E. grandis* Hill ex Maiden, *E. globulus* Labill, *E. camaldulensis* Dehnh, *E. urophylla* Blake and their hybrids, are used to increase the supply of wood to meet industrial and domestic demands (Grattapaglia and Kirst 2008). Eucalypts have been adopted for plantation forestry in more than 100 countries worldwide accounting for more than 20 million planted ha (Iglesias-Trabad et al. 2009). Small fibres, smoothness, brightness and low tensile strength are characteristics that make eucalypts reliable for use in the kraft pulp industry, which is largely based on two species, *E. globules* and *E. grandis,* and their hybrids. Besides the production of pulpwood, these species and their hybrid clones are also used for timber, firewood, shelter and in furniture industries (Pinto et al. 2002, 2016). As eucalypts exhibit their ability to grow in a wide range of environments and soils, it is expected that areas occupied by these fast-growing species will continue to expand worldwide in the following decades.

Breeding of eucalypts for industrial plantation has the objective of improving target traits such as volume growth, wood density, and pulp yield, as well as to increase tolerance to both biotic and abiotic stresses. The breeding scheme involves recurrent selection cycles from which elite trees are derived for the establishment of seed orchards and/or to be tested as potential commercial clones (Grattapaglia and

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Kirst 2008). The vegetative propagation of selected clones plays a pivotal role in the exploitation of breeding programs by capturing both additive and non-additive genetic variance. *Eucalyptus* vegetative propagation is mainly carried out through the rooting of cuttings. However, this strategy is limited by the heterogeneous rooting ability among genotypes, which normally tends to decrease as the age of the parent tree from which the cuttings are collected increases (Mankessi et al. 2010). Clonal propagation through in vitro technologies can provide a complementary alternative to the vegetative propagation of the species. Axillary shoot multiplication has been the most frequently used methods (Pinto et al. 2011; Aggarwal et al. 2016). Unfortunately, the low rooting rates recorded in conventional propagation are also recorded by rooting microcuttings of the same genotypes (Assis et al. 2004). The advantage of the in vitro procedure is related to the ability to modify the culture conditions to obtain acceptable rooting rates for commercial purposes. We studied the organogenic response of an *E. saligna* × *E. maidenii* hybrid genotype and two *E. globulus* genotypes. Plant material to initiate the cultures was collected from selected 12-year-old mother trees. Initial rooting rates achieved were 75, 16, and 37%. These figures were clearly improved to 100, 76, and 86%, respectively, by appropriate modifications of the culture conditions, especially by the lack of NH_4NO_3 in the rooting medium (unpublished results). According to Correia et al. (2011) the role of the genotype in vegetative propagation should be interpreted carefully, as culture conditions can be optimised and the genotypes behave differently under different conditions.

Somatic embryogenesis (SE) is another method of vegetative propagation. In fact, SE of forest trees offers the potential for propagation on a scale that was not previously possible with other vegetative propagation methods (Thompson 2015). Applying this technique in clonal forestry has multiple advantages, including high multiplication rates, potential for scale-up and delivery via bioreactors, application of synthetic seed technologies, production of suitable target tissue for gene transfer, and the maintenance of cultures by low growth conditions or cryopreservation (Pinto et al. 2016). Somatic embryogenesis plays a key role in the concept of Multi Varietal Forestry (MVF), which is defined as the clonal deployment of tested tree varieties in commercial plantation forestry, which may dramatically increase forest productivity (Park and Bonga 2010). MVF is currently applied at a commercial scale in Canada with several conifer species and the genetic gain per unit time is expected to notably increase if MVF is integrated with genomic selection (GS) and SE (Park et al. 2016). Since GS can provide genetic information of individuals at a very early stage, it can be used to preselect genotypes to be included in MVF. Eucalypt species would be considered good candidates for the application of the combination of MVF and genetic selection. In 2014, the genome of *E. grandis* was sequenced (Myburg et al. 2014) providing a powerful tool to accelerate comparative biology, breeding and biotechnology (Marcon et al. 2015; Grattapaglia et al. 2015; Gion et al. 2015; Ribeiro et al. 2016). As SE is the other tool required to vegetatively propagate selected material, a brief revision of the state of the art in the process is given.

The efforts carried out on the development of consistent SE procedures in eucalypts were recently reviewed by Pinto et al. (2016). Somatic embryogenesis has been reported for *E. grandis*, *E. citriodora*, *E. gunnii*, *E. nitens*, *E. globulus*, *E. tereticornis*, *E. camaldulensis* and several hybrids (Chauhan et al. 2014; Pinto et al. 2013; Corredoira et al. 2015). In most of these reports, embryogenic cultures were initiated from zygotic embryos or young seedlings and the responses achieved are related to low induction rates and low embryo germination frequencies. Reports on the induction of SE from tissues of mature trees are scarce but a reproducible procedure for *E. globulus* and the hybrid *E. saligna* × *E. maidenii* has been developed (Corredoira et al. 2015). However, as in other species (Ballester et al. 2016), most of these publications are based on single experiments with different species, or experiments based on a single genotype, or do not provide sufficient data to allow replication of the work. According to Thompson (2015), there are many factors limiting the scale-up of SE that normally affect both gymnosperms and angiosperms because initiation and, to a lesser extent, maturation and germination rates are under strong additive genetic control (Bonga et al. 2010).

The objective of the present review is the description of SE protocols that are currently applied to eucalypts. One part of the protocol will deal with the induction of SE using zygotic embryos as the target material, but induction from material collected from mature trees will also be described. For practical reasons, the review will focus on *E. globulus* because the most consistent and reproducible reports of SE have been carried out in this species (Pinto et al. 2016). The difficulties encountered during the SE procedure will be highlighted and recommendations to improve the protocol will be given.

2 Materials

The regeneration procedure includes three steps: (1) induction of somatic embryogenesis from different explants, (2) proliferation of embryogenic lines, and (3) somatic embryo germination and plantlet conversion.

2.1 Materials

1. Plant materials as source of initial explants: immature seeds, seedlings or axillary shoot cultures established from epicormic shoots forced from branch segments of adult trees.
2. Ultrapure water Milli-Q, distilled water, ethanol (96 and 99°), Benlate®, hydrogen peroxide, 1000–5000 mL Erlenmeyer flasks.
3. Mineral salts, plant growth regulators (PGRs), organics and other substances for media preparation: 6-benzyladenine (BA), naphthalene-acetic acid (NAA), gibberelic acid (GA_3), Gelrite, Vitro agar (Pronadisa, Spain), sucrose, silver

thiosulphate (STS; sodium thiosulfate, silver nitrate), arabic gum from acacia tree (Sigma, St. Louis, USA, G-9752), and casein hydrolysate (CH) (see Table 1).
4. Pipettes (1–25 mL), air-displacement piston pipettes (2–1000 µl), micropipettes, and single use filters (22 µm).
5. Precision balance and autoclave.
6. Culture vessels of different types: 90 × 15 mm-diameter Petri dishes and test tubes (150 × 25 mm). Filter paper discs (Whatman grade 181). Parafilm®.
7. Laminar-flow cabinets with ultraviolet light. Glass bead sterilizer.
8. Stereoscopy microscopes, forceps, and scalpels.
9. Rotary microtome, transmission light microscope, and chemicals for microscopy: formalin, glacial acetic acid, n-butanol, paraffin wax, safranin, fast green, periodic acid-Schiff (PAS) kit, naphtol blue-black.
10. Fungicidal solutions: Previcur® (66.5% propamocarbe-hydrochloride, Bayer®) and Derosal® (60% carbendazamine, Bayer®).
11. Culture growth chambers and phytotron.

Table 1 Culture media used in the somatic embryogenesis of *E. globulus*. All concentrations are in g L^{-1}, except those of PGRs that are in µM

Media components	Embryo induction medium for ZE (M1)	Shoot proliferation medium (M2)	Embryo induction medium for leaf/apex (M3)	Embryo proliferation medium (M4)	Liquid germination medium (M5)
Basal medium	MS	MS	MS	MS	MS
Sucrose	30	20	30	30	30
CH	–	–	0.5	0.5	0.5
Citric acid	–	0.01	–	–	–
Ascorbic acid	–	0.01	–	–	–
Folic acid	–	0.001	–	–	–
Arabic gum	–	–	0.04	–	–
Gelrite	2.5	7	–	–	–
Vitro agar	–	–	6	6	6
BA	–	0.44	–	–	0.44
Picloram	–	–	40[a]	–	–
NAA	16.11	0.054	16.11[a]	16.11	–
GA_3	–	–	–	–	1.44
pH	5.8	5.6–5.7	5.6–5.7	5.6–5.7	5.6–5.7

ZE zigotic embryos
[a]For somatic embryo induction in leaf and shoot apex explants, M3 medium can be supplemented with NAA or Picloram

2.2 Culture Media

Regardless of the type of explants used for initiation of the embryogenic process, the basal medium is based on the MS macro and micronutrients and vitamins (Murashige and Skoog 1962). This basal medium is used throughout the protocol, but different additives are added. In Table 1, the media used for plant regeneration by somatic embryogenesis of *E. globulus* are summarized.

Adjust the pH of the different media with 0.5 N NaOH or 1 N HCl and dispense 16.5 mL in test tubes or 25 mL in 90 mm- diameter Petri dishes. Autoclave for 20 min at 121 °C and 1×10^5 Pa (1.1 kg cm^{-2}).

3 Protocol of Somatic Embryogenesis

3.1 Induction of SE from Explants of Juvenile Origin

Different explants from juvenile origin have been used to initiate the embryogenic process in *E. globulus*. Cotyledons and hypocotyls of 3-day-old seedlings, leaves and stems of 2-month-old plants and entire mature zygotic embryos have been used. Zygotic embryos as explants, however, have yielded the most consistent and repetitive responses thus far (Pinto et al. 2002, 2008, 2013). The following aspects of the protocol refer to the culture of mature zygotic embryos:

1. Collect seeds of *E. globulus* Labill., preferentially half-sib seeds from a breeding program. The collection of different seed families is recommended as the embryogenic response is genotype-dependent.
2. Sterilise the seeds with a mixture of absolute ethanol:hydrogen peroxide 30% (v/v) for 15 min.
3. Wash the seeds twice in sterile distilled water for 10 min each, and rinse them with 0.1% (w/v) Benlate® for 15 min.
4. Leave the seeds to imbibe overnight in distilled water.
5. Under aseptic conditions, remove the seed coat and initiate the cultures from entire mature zygotic embryo explants.
6. Place the explants on 90-mm diameter Petri dishes (10 explants per dish) on embryo induction medium (M1) consisting of MS salts with vitamins supplemented with 30 g L^{-1} sucrose, 2.5 g L^{-1} Gelrite® and 16.11 μM NAA (Table 1).
7. Keep the cultures in the darkness at 21 ± 1 °C for 3 weeks.
8. During this period, germination of entire zygotic embryos may be observed in the induction medium, but the germination process may stop 2 weeks after inoculation.
9. After the 3-week induction period, transfer the explants monthly to the expression medium comprising of M1 medium (Table 1), but without PGRs, for a total period of 4 months.

10. At the end of the 4-month period, calculate the embryogenic potential by determining the percentage of explants showing SE, the total number of somatic embryos and the type (globular or cotyledonary-stage) of embryos formed.

In general, somatic embryos are formed indirectly from the embryogenic calli but, occasionally, direct somatic embryogenesis also occurs from the upper surface of hypocotyls. Embryogenic calli are identified as compact and white-brownish (Pinto et al. 2002).

In most cultures of eucalypts for SE induction, the development of roots and shoots together with somatic embryos is typical. Careful observations including histological study (see subsection 3.3) are required to distinguish the different organs (Dobrowolska et al. 2016).

3.2 Induction of SE from Explants of Adult Origin

Studies leading to the induction of SE from explants of adult material are scarce in eucalypts and, to the best of our knowledge, are limited to those reported about 20 years ago for Qin and Kirby (1990) in adult *E. grandis* genotypes, and Termignoni et al. (1998) working with *E. saligna* Smith and *E. dunnii*. In both reports, insufficient information was given to reproduce the protocols. Recently, we defined a repetitive and consistent protocol for the induction of SE in material collected from selected 12-year-old trees of *E. globulus* (2 genotypes) and one genotype of the hybrid *E. saligna* × *E. maidenii* (Corredoira et al. 2015).

Leaves and shoot tip explants collected from axillary shoot multiplication cultures of the species were used to initiate the embryogenic process. In vitro material was previously used for induction of SE with juvenile material of *E. grandis* (Watt et al. 1991) or for the establishment of cell suspension cultures in *E. grandis* and *E. grandis* × *camaldulensis* (Blakeway et al. 1993).

Consequently, to develop the protocol that will be described below, a shoot multiplication culture of the material in question is required. Organogenesis in eucalypts is, in most cases, not problematic and there is sufficient information on the topic (Aggarwal et al. 2016).

3.2.1 Explant Preparation

1. Stock shoot cultures should be stabilised and maintained by periodic subculture before being used in SE induction experiments (Fig. 1a).
2. Multiply axillary shoot cultures every 3–4 weeks on shoot proliferation medium (M2) (Table 1). Shoots (1 cm long) are cultured on test tubes with 16.5 mL of M2 medium.

3. Stock cultures are incubated in a growth chamber with a 16-h photoperiod (provided by cool-white fluorescent lamps at a photon flux density of 50–60 μmol m^{-2} s^{-1}) at 25 °C.

3.2.2 Induction of Somatic Embryos

1. Isolate the shoot apex (1.5–2 mm long) comprising the apical meristem 2–3 leaf primordia and the two most apical expanding leaves from the first (leaf 1) and second (leaf 2) nodes in the apical region of the shoots in an active state of growth.
2. Place 10 shoot apices (horizontally oriented) and 10 leaf explants (abaxial side down) each in 90-mm diameter Petri dishes containing 25 mL of M3 medium (Table 1) consisting of MS salts and vitamins, 30 g L^{-1} sucrose, 6 g L^{-1} Vitro agar, 0.5 g L^{-1} casein hydrolysate, 40 μM picloram and 40 mg L^{-1} of arabic gum.
3. Incubate the cultures in darkness at 25 °C for 8 weeks.
4. After the 8-week period, the initial proliferated explants resemble a watery callus of friable consistency, which is initially yellow and then may turned necrotic, dark brown and disaggregated (Fig. 1b). With the help of a stereo-microscope, identify the embryogenic tissue: white-translucent nodular structures that arise from the watery callus. These structures are white-translucent, nodular-ovoid, cup-shaped and curved (Fig. 1c).

The embryogenic response is defined as the presence of embryogenic structures and/or somatic embryos (torpedo-cotyledonary stage) in the initial explants. Somatic embryos are identified and isolated, which are easily detached from the initial explants, and characterised by a clear bipolarity with development of root and shoot poles and two small leafy cotyledons.

Alternatively, picloram may be substituted for 16.11 μM of NAA (Fig. 1d), although the embryogenic rates were lower than with picloram culture.

3.3 Histological Study

Histological analysis could be a necessary step to confirm somatic embryogenesis and to determine whether the plantlets regenerated have an organogenic or embryogenic origin. For histological examination, explants (for example nodular structures and somatic embryos) were processed as follows:

1. Fix the samples in a FAA solution (formalin, glacial acetic acid and 50% ethanol (1:1:18, v/v/v) for 48 h.
2. Dehydrate the samples through a graded n-butanol series, 48 h each (Jensen 1962).

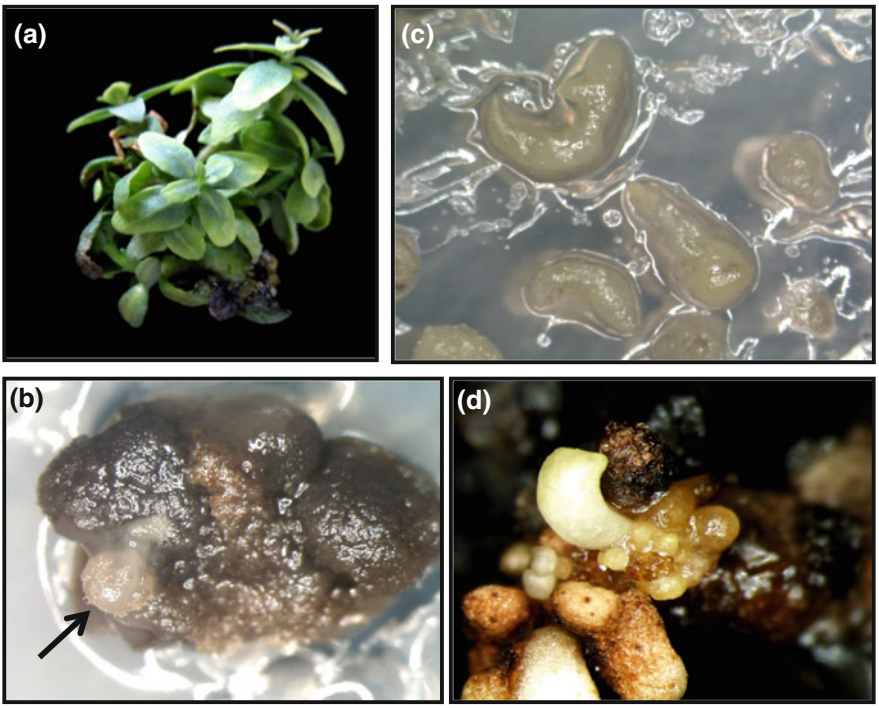

Fig. 1 Induction of somatic embryogenesis in leaf and shoot apex explants isolated from axillary shoot cultures established in vitro from *E. globulus* trees. **a** Axillary shoot used as source of initial explants. **b** Nodular embryogenic structure (arrow) arising from a watery callus tissue formed in a leaf explant after 8 weeks of culture in picloram-containing induction medium. **c** Individual embryogenic structures isolated from a watery callus with a white-translucent and shiny appearance. **d** Somatic embryos initiated in an apex explant after 8 weeks of culture in NAA-containing induction medium

3. Embed the dehydrated samples in paraffin wax.
4. Obtain serial sections of tissues of 8 μm thickness using a Reichert-Jung rotatory microtome.
5. Stained sections with safranin-fast green for general examination (Jensen 1962) and periodic acid-Schiff (PAS)-naphthol blue-black to detect starch and other insoluble polysaccharides and total proteins (Feder and O'Brien 1968).
6. Mount the stained sections with Eukit® and examine them using a transmission light microscope.

To confirm the embryogenic character, somatic embryos should show a bipolar organization with root and shoot apical meristems, and closed vascular tissue that bifurcated into small cotyledons.

3.4 Secondary Embryogenesis

Embryogenic capacity can be maintained by secondary embryogenesis (Fig. 2a). The procedure described here refers to somatic embryos of adult origin, as described by Corredoira et al. (2015).

1. Small clusters of globular-stage embryos (2–4 embryos) were used as subcultured explants.
2. Culture these explants in 90-mm diameter Petri dishes containing 25 mL embryo proliferation medium (M4) consisting of MS salts and vitamins, 0.5 g L^{-1} casein hydrolysate, 30 g L^{-1} sucrose, 6 g L^{-1} Vitro agar and 16.11 μM NAA (Table 1).
3. Incubate the cultures at 24–25 °C in the dark. Maintain darkness until the cotyledonary stage of embryos is reached.

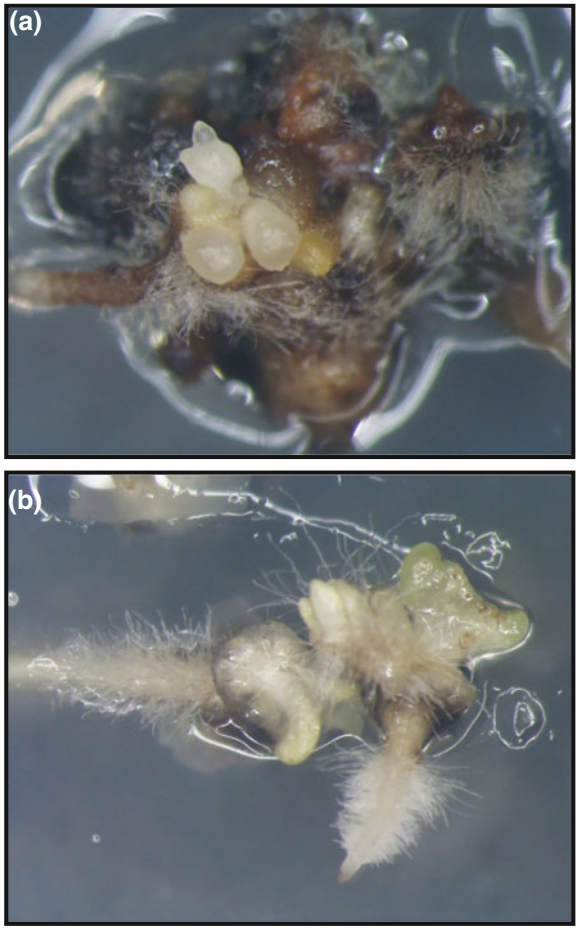

Fig. 2 Proliferation by secondary embryogenesis of somatic embryos. **a** Secondary embryos developing in proliferation medium with NAA. **b** Secondary somatic embryos showing precocious germination

4. Subculture the small clusters onto fresh proliferation medium each 8–12-weeks.
5. Estimate the embryogenic capacity by calculating the percentage of subcultured clusters that generated secondary embryos, and by recording the number of secondary embryos (showing bilateral symmetry) produced per 90-mm diameter Petri dish.
6. Precociously germinating somatic embryos may be found in the embryo proliferation medium (Fig. 2b). The addition of an ethylene inhibitor such as STS at a concentration of 20 μM could improve the embryo proliferation (Martínez et al. 2015). STS should be filter-sterilised before being added to the autoclaved proliferation medium.

3.5 Plantlet Conversion

As for other woody plants, plant recovery from somatic embryos in *Eucalyptus* species is a difficult step and has only been described in a small number of embryogenic cultures, with different levels of success (Pinto et al. 2016). In *E. globulus*, two procedures can be used to obtain plants from somatic embryos.

1. Transfer somatic embryos to germination medium consisting MS salts and vitamins supplemented with 30 g L^{-1} sucrose and 2.5 g L^{-1} Gelrite®.
2. Culture the somatic embryos under dim light conditions (first 5 days under 5.70 ± 2 μmol m^{-2} s^{-1}) at 24 ± 1 °C, and then light exposure was increased gradually.
3. Isolate cotyledonary stage somatic embryos, transfer them to fresh germination medium and re-incubate under the conditions described above.

When precocious germination of somatic embryos was observed (Fig. 2b), the use of liquid germination medium is highly recommended to avoid drying.

1. Place cotyledonary stage embryos (6–8 embryos) on two filter paper discs (Whatman grade 181) in 90-mm diameter Petri dishes containing 10 mL of liquid germination medium (M5) consisting of MS salts and vitamins supplemented with 30 g L^{-1} sucrose, 0.44 μM BA and 1.44 μM GA_3 (Table 1; Fig. 3).
2. Incubate the cultures in a growth chamber with a 16-h photoperiod (provided by cool-white fluorescent lamps at a photon flux density of 50–60 μmol m^{-2} s^{-1}) at 25 °C for 6 weeks.

In general, enlargement and greening of hypocotyl and cotyledons followed by root growth typically occur in most of the somatic embryos; however, embryo plantlet conversion with both root and shoot development was only observed in a few instances (Corredoira et al. 2015). As an alternative when low conversion rates are obtained, the shoots derived from germinating somatic embryos can be used to establish axillary shoot culture lines. For this procedure, excise the shoots from germinating embryos and subculture them on shoot proliferation medium, according to the method previously defined under 3.2.1.

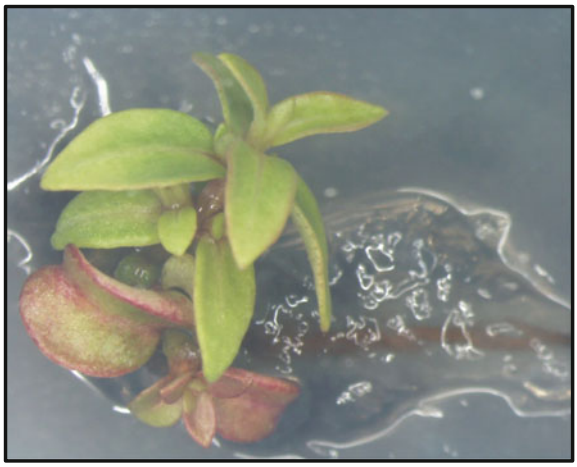

Fig. 3 Plant regeneration from a somatic embryo after 6 weeks of culture in liquid germination medium and 4 weeks in shoot proliferation medium

3.6 Acclimatisation of Plantlets

Acclimatisation of *E. globulus* has been reported by Pinto et al. (2011).

1. Transfer the emblings (minimum 2 cm long) to pots containing sterilised peat: perlite (3:2).
2. Place the pots in a phytotron at 24 °C with a photoperiod of 16 h and a light intensity of 480 μmol m^{-2} s^{-1}. Initial relative humidity should be higher than 95%, decreasing weekly to reach 50–60%.
3. Water the pots weekly with a commercial solution of 5 mL L^{-1} Complesal-Calcium® (Agrevo).
4. Calculate the percentage of surviving plants after two weeks in phytotron.
5. Assess the development of leaves and internodes and annotate any sign of stunting, hyperhydricity or chlorosis (Pinto et al. 2011).

To prevent fungal infections, add Previcur® (1.5 mL L^{-1}) and Derosal® (0.75 g L^{-1}) occasionally to both plantlets and soil solutions.

4 Research Prospects

In spite of the considerable progress that has been made in the development of somatic embryogenesis methods for *E. globulus* and other eucalypt species, the practical application of the procedure is far from being achieved. Taking into consideration the great industrial interest of the species, much more effort should be applied to clarify the different steps of the embryogenic process. As defined in the present review, the induction of SE is feasible not only for material of juvenile origin but also from material collected from mature eucalypt trees. In the latter, the

use of axillary shoot cultures as a source of initial explants is highly recommended, as this provides uniform explants, the supply of an unlimited number of explants is guaranteed all year around, and no re-sterilisation of the material is required. The proliferation step of the embryos may be difficult to interpret, as somatic embryos at different stages of development, precociously germinated embryos, roots and even adventitious shoots may simultaneously develop. Research should address this step to obtain only somatic embryos and to avoid, if possible, precocious embryo germination. As in many other woody species, the maturation and germination steps are poorly optimised and, consequently, low rates of plantlet conversion have often been reported. Since these steps are not optimised, and in order to mitigate the low plantlet conversion rates achieved during the embryogenic process, we highly recommend the use of axillary shoot proliferation of the germinated embryos as a realistic step to increase the plant production. Given the economic importance of these species, additional research is needed to refine the embryogenic process and realise its full potential. In addition, investigations that combine molecular genetics, somatic embryogenesis and genetic transformation are probably the most appropriate way to accelerate the improvement processes in the species.

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Cryopreservation of Hybrid *Pinus elliottii* × *P. caribaea*

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
ABA	Abscisic acid
BAP	6-benzylaminopurine
DMSO	Dimethyl sulfoxide
ECL	Embryogenic cell line
EM	Embryonal mass
mLV	Modified Litvay's medium
MVF	Multi-varietal forestry
mLV	Modified Litvay medium
PEG	Polyethylene glycol
Pi	Propidium iodide
PGR	Plant growth regulators

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SE Somatic embryogenesis
PEE *Pinus elliottii* var. *elliottii*
PCH *Pinus caribaea* var. *hondurensis*

1 Introduction

The interspecific hybrid between *Pinus elliottii* var. *elliottii* (PEE) (slash pine) and *Pinus caribaea* var. *hondurensis* (PCH) (caribbean pine) was first developed in the mid 1950's in Queensland (Australia) for producing a high-yielding hybrid with improved characteristics and best adapted to that region (Slee 1970). Several field trials have confirmed that the performance of the hybrid *P. elliottii* var. *elliottii* × *P. caribaea* var. *hondurensis* (PEE × PCH) had a superior performance than its parental species. In Queensland, the F1 hybrid had improved growth and stem straightness, together with small branches, wind firmness, and superior wood quality (Nikles 2000; Trueman 2006). The hybrid superiority derives from the complementary recombination of parental traits—faster growth, superior branch quality, uniform wood of PCH combined with higher wind firmness, better adaptability to wet sites, higher wood density and stem straightness of PEE (Dieters and Brawner 2007).

Due to its high productivity and good performance in a broad range of soil and environmental conditions, there has been an increasing industrial demand for the hybrid PEE × PCH with consequent expansion of the planted area to regions that were traditionally planted with PEE. For example, the hybrid PEE × PCH is, currently, commercially exploited in several regions of Brazil, Argentina and South Africa (Gauchat et al. 2005; Nikles 2000; van der Sijde and Roelofsen 2010). The primary use of this hybrid is in wood industry, particularly, for structural timber, veneer and plywood products (Shepherd et al. 2002), but it has also gained relevance in the gum-resin industry, especially in Brazil.

Given its increasing importance to forestry industry, the PEE × PCH has been involved in several breeding programs aiming to achieve high-productive clonal varieties adapted to each location. Besides pine breeding program from Queensland, in Argentina a hybridization program was also established to obtain locally improved and adapted hybrid clones for growth rate and stem form (Gauchat et al. 2005; Cappa et al. 2013). Private companies in Brazil are also investing in the development and selection of improved clones regarding better growth rate, wood properties and higher resin production. In South Africa, studies developed by Roux et al. (2007) on Pinus species and their hybrids showed that hybrid PEE × PCH was more tolerant to infection by *Fusarium circinatum* (the pitch canker fungus) indicating its potential for breeding concerning tolerance to pitch canker in this country.

Cryopreservation is an effective tool for long-term conservation of important and unique plant genotypes outside their natural habitats, useful for breeding programs, and playing also an important issue concerning the conservation of plant biodiversity.

The Cryosystems allow the long- term storage of plant genetic resources at low temperature in liquid nitrogen (−196 °C) or in vapor phase nitrogen (−150 °C), when all cellular divisions and metabolic processes are suspended to minimum levels. Under controlled environment the plant material such as somatic/zygotic embryos, shoots tips/axillary buds, cell suspensions, callus, pollen, among others, can be stored, remaining viable during decades. Cryopreservation thus, allows a safe, minimal maintenance and cost-effective long-term conservation of plant genetic resources, avoiding the risks faced by field populations, which include the exposure to unknown climatic factors and diseases.

Cryopreservation of somatic embryogenesis is a key technology for the clonal selection and commercial production of implementing multi-varietal forestry (MVF). This technology ensures the availability of juvenile material, overcoming the maturation problems associated to the trees plantations and providing biological material genetically stable (Lelu-Walter et al. 2016; Park et al. 2016). The EMs cryopreservation is a crucial tool to avoid the loss of regeneration ability, prevent the potential loss of embryogenic capacity and putative somaclonal variation frequently found in long-term subcultures, (Latutrie and Aronen 2013; Klimaszewska et al. 2016).

Besides the several plant cryopreservation methods, such as encapsulation, vitrification, dehydration, the controlled rate cooling or slow-cooling remains the most common approach for cell suspensions, callus and embryogenic callus, with higher regrowth rates, namely in conifers (Ozudogru and Lambardi 2016). This chapter describes the protocol to cryopreserve somatic embryogenic cultures of the interspecific hybrid PEE × PCH, through the controlled rate cooling method, and reports a methodology to evaluate the whole genome stability at the ploidy level by flow cytometry.

2 Cryopreservation

2.1 Plant Material

Plant source material from somatic embryogenesis: The proliferating embryonal mass (EM) of the hybrid PEE × PCH is used as starting plant material for the cryopreservation procedure. This EM is initiated from immature zygotic embryos aseptically removed from megagametophytes, and sub-cultured bi-weekly on fresh Proliferation medium (PM) (Table 1). Three to four-month-old EMs are used for the cryopreservation experiments.

Table 1 Composition of culture media for *P. elliottii* var. *elliottii* × *P. caribaea* var. *hondurensis*. Concentrations in mg L^{-1}

Components	Concentration (mg/L)			
	P Proliferation medium	MM Maturation medium	GM1 Germination medium 1	GM2 Germination medium 2
Inorganic macroelements				
$CaCl_2$	8.31	8.31	4.16	4.16
KH_2PO_4	170	170	85	85
KNO_3	950	950	475	475
$MgSO_4.7H_2O$	925	925	462.5	462.5
NH_4NO_3	825	825	412.5	412.5
Micronutrientes				
$CoCl_2$	0.125	0.125	0.063	0.063
$CuSO_4.5H_2O$	0.5	0.5	0.25	0.25
H_3BO_3	31	31	15.5	15.5
KI	4.15	4.15	2.075	2.075
$MnSO_4.H_2O$	21	21	10.5	10.5
$Na_2MoO_4.2H_2O$	1.25	1.25	0.63	0.63
$ZnSO_4.7H_2O$	43	43	21.5	21.5
$Na_2EDTA.2H_2O$	37.3	37.3	18.65	18.65
$FeSO_4.7H_2O$	27.8	27.8	13.9	13.9
Organics				
Mio-inositol	100	100	50	50
Ac. Nicotínico	0.5	0.5	0.25	0.25
Piridoxina HCl	0.1	0.1	0.05	0.05
Tiamina HCl	0.1	0.1	0.05	0.05
Casein hydrolysate	1000	1000	500	500
L-glutamine	500	500	250	250
Plant growth regulators				
BAP	1	–	–	–
2,4-D	2	–	–	–
ABA	–	10.57	–	–
Other additions				
Activated charcoal	–		2 500	–
Sucrose	20,000	60,000	20,000	20,000
Gelrite	4000	9000	6000	6000

Notes The pH of all media is adjusted to 5.8
Amino acids and ABA are filter sterilised and added to cooled medium
Basal media based on modified Litvay's et al. (1985) medium (mLV), Klimaszewska et al. (2001)

2.2 Cryoprotection

A combination of non-penetrating and penetrating solutes with colligative actions is applied as chemical cyoprotection to the cell suspension cultures, avoiding the cryoinjuring caused by the ice crystal formation, and subsequently the cell disruption and death (Martinez-Montero and Harding 2015).

A good physiological and a healthy state of the starting plant material are essential to the cryopreservation success. About 3.16 g of actively growing EM (harvested 7 days after transfer onto fresh PM medium) is added to 12.8 mL liquid PGR-free PM medium. To break up the EM clumps into a fine suspension, masses are carefully disaggregated using a sterile pipette tip and shaking vigorously the suspensions.

The embryogenic cell suspension cultures are pretreated with the non-penetrating dehydrative cryoprotectant sucrose, over a 2-day period. On the first day, 1.42 mL filter-sterilized sucrose stock solution (2 M) is added to the suspension. During this period the cultures are incubated on a rotary shaker at 100 rpm, at 23 ± 2 °C, in the darkness.

On the second day sucrose stock solution is added dropwise over a period of 30 min to the final concentration of 0.4 M. The suspensions are kept for 10 min on ice for cell sedimentation, after which 3.95 mL of the supernatant liquid is poured. Filter sterilized and cooled PSD solution [20% PEG 4000, 20% sucrose and 20% DMSO (v/v)] is added dropwise over a period of 30 min, to the final concentration of 5% for each component. The suspensions must be stirred after each addition of PSD solution, and finally stirred for 1 h, on ice. Aliquots of 1.8 mL of the pretreated embryogenic suspension can be dispensed into at least 7 cryovials. Untreated and non-frozen samples should be used as controls.

2.3 Cooling

In order to restrain the intracellular ice formation, the EMs are submitted to a controlled rate cooling methodology. The pretreated embryogenic suspension dispensed into cryovials are placed on an alcohol-free cell freezer container (CoolCell, by BioCision), kept at −80 °C. The CoolCell ensures higher vial consistency and reproducible controlled-rate freezing (−1 °C/min), when compared with Mr. Frosty container.

After 24 h at −80 °C, the cryovials are plunged directly into a cryocontainer that allows the storage in vapor phase (−150 °C). To avoid cross contamination the storage in vapor phase is the safest ultra-low temperature system, when compared to the liquid nitrogen (−196 °C) Cryosystems.

2.4 Thawing

To avoid the ice re-crystallization, the sample rewarming must be performed as quickly as possible. After the cryostorage, the samples removed from the cryocontainer must be immediately warmed in a 45 °C water-bath for 3–4 min, until all ice is melted. The cryovials must be transferred onto ice to quickly decrease the temperature. The surface of cryovials are sterilized with 70% ethanol, dried in a laminar flow hood before the embryogenic suspensions are transferred to a disk of sterile filter paper (55 mm Whatman No. 2). To avoid the potential toxicity of the cryoprotectors, the excess of cryoprotective liquid is adsorbed from the filter paper, using sterile paper towels. The filter papers with cultures are transferred to PM Medium, maintained at 23 ± 2 °C in the dark, and thereafter EMs are subcultured every 2 weeks.

Concerning the Cryogenic storage and the regrowth efficiency it is important to thaw and recovery at least 3 cryovials per genotype, to confirm the efficiency of the cryopreservation procedure.

3 Cryopreserved Cell Viability and Regeneration of Somatic Plants Recovered from Cryopreserved EMs

The recovered EMs are monitored by light microscopy observations, to confirm the cryopreserved cell viability. Samples from the different embryogenic cell lines are gently squashed in acetocarmine. The stained white masses render a typical EM, having aggregates of meristematic cells (small, round and dense cytoplasmic cells) together with elongated and highly vacuolated cells, forming a typical embryonal tissue.

It is also important to evaluate the maturation and germination ability of the recovered EMs. Embryogenic suspensions are performed with 200 mg of cryo and non-cryopreserved EMs 7 days after subculture, into 3 mL of liquid PGR-free proliferation medium. One mL of suspension is spread on a filter paper (Whatman No. 2, 55 mm) previously placed on sterile paper towels to drain excess liquid. EMs on the filter paper are subcultured in MM medium (Table 1), and incubated in the dark and in a growth chamber at 23 ± 2 °C. Cotyledonary embryos are transferred to GM1 medium (Table 1) during 1 week in the dark, then one week in subdued light and 3 weeks in normal light in a growth chamber, at 23 ± 2 °C, for a 16/8-h (day/night) photoperiod, under a photosynthetic photon flux density (PPFD) of 50 ± 10 μmol m^{-2} s^{-1}. The germinated embryos are transferred to GM2 into flasks in the light conditions, at 23 ± 2 °C. Finally, after 4 weeks the somatic plants' roots are washed to remove medium debris, and emblings transferred to ex-vitro conditions of peat:perlite:vermiculite (8:1:1), in a growth chamber. The growth chamber relative humidity was progressively reduced (from ~95 to ~45%) during 10 days, avoiding air circulation.

Note: All culture manipulations must be performed in aseptic conditions using laminar flow hood and sterile material.

4 Ploidy Stability Analysis

Some studies have shown that pine embryogenic masses become unstable in culture, with loss of maturation and regeneration ability, especially when these are maintained for long periods (Latutrie and Aronen 2013; Nunes et al. 2017a). Whilst at cryogenic temperatures the embryogenic cultures can be theoretically maintained indefinitely without genetic alterations, the cryopreservation procedure exposes the EMs to different stresses that may cause cryoinjury (Harding 2004), putatively contributing to genetic/ploidy instability.

Here we describe a simple procedure to evaluate the whole genome stability at ploidy level by Flow Cytometry. Fresh cryo and non-cryopreserved EMs under proliferation, and somatic plants are used as plant starting material, for the ploidy analyses. The first step is the release of nuclei by mechanical homogenization, into a nuclear isolation buffer. These nuclear suspensions are prepared by chopping approximately 50 mg of plant material using a sharp razor blade, in Woody Plant Buffer pH 7.5 (Loureiro et al. 2007) (Table 2). The buffer is important not only to ensure the release of a large number of nuclei but also to keep nuclei stability, preventing nuclear aggregation and DNA protection (Doležel and Bartoš 2005). The nuclear suspensions are filtered through a 50 μm nylon filter, to remove cell fragments and large debris. After filtration, the nuclei must be stained with a fluorochrome, preferably propidium iodide (PI) that intercalates into double-stranded nucleic acids, and is excited by a 488 nm argon laser (Doležel et al. 1992). So, 50 μg/mL of PI (Fluka) is added to the nuclei suspension. A pre-treatment with 50 μg/mL RNAase (Sigma) added to the samples is also performed for an accurate DNA measurement. Samples are incubated for 5 min at 4 °C, before the analysis in the flow cytometer. Several brands and models of flow cytometers equipped with a 488 nm laser can be used, and for example, a Beckman Coulter (Beckman Coulter Life Sciences, USA), or an Attune Acoustic Focusing

Table 2 Composition of nuclear isolation buffer, the woody plant buffer (Loureiro et al. 2007)

Components	Concentration
Tris–HCl	0.2 M
$MgCl_2$	4 mM
EDTA Na_2	2 mM
NaCl	86 mM
Sodium metabisulfite	10 mM
PVP-10	1%
Triton X-100	1% (v/v)

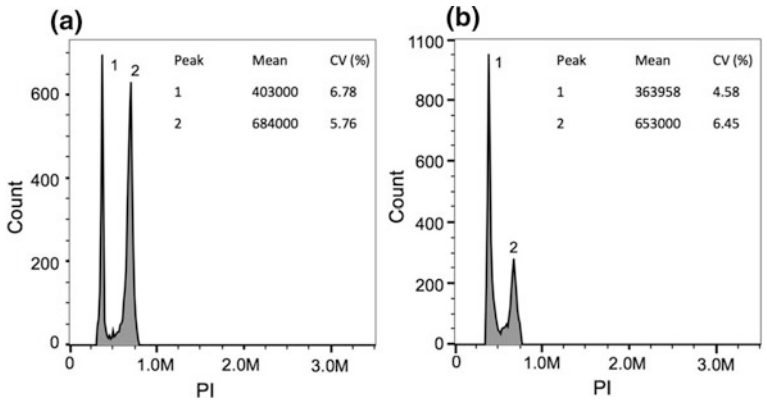

Fig. 1 Histograms of relative fluorescence intensity (PI) obtained for hybrid *Pinus elliottii* × *P. caribaea* nuclei: **a** from EM; **b** from recovered EM. Peak 1—*Vicia faba*; Peak 2—hybrid PEE × PCH; CV—coefficient of variation

Cytometer (Life Technologies Applied Biosystems, Vic, Australia) provided very reliable analyses for this hybrid (Fig. 1).

For the data analysis the use of a software as FlowJo (Tree Star Inc., Ashland, OR, USA) is advisable. The ploidy levels and putative occurrence of aneuploidy or polyploidy are identified by analysing the G_0/G_1 peaks position and/or appearance of new G_0/G_1 peaks.

For absolute estimations of nuclear DNA content, it should be used an internal standard, which consists of the nuclei isolated in the same way from a standard species (e.g. leaves), which has a well-known genome size (pg), such as *Vicia faba* cv. Inovec. Best results are achieved using the nuclei suspension combined with those of the sample (internal reference standard).

5 Conclusions and Future Perspectives

Clonal forestry is one of the main approaches used for the establishment of the genotypes from the elite PEE × PCH hybrid, first established in Queensland. The low production of seeds by the F1-hybrid promoted the use of vegetative multiplication of offspring F1 (Schenone and Pezzutti 2003) through the propagation of rooted cuttings and tissue culture (Trueman 2006; Shepherd et al. 1999). For the successful integration of vegetative propagation in breeding programs, SE and somatic embryos cryopreservation can be an important tool (Park et al. 2016).

The protocol presented here is an efficient procedure to cryopreserve PEE × PCH embryogenic cell lines (Fig. 2), representing a crucial tool to scale up the production of superior genotypes via SE. However, concerning the integration of hybrid cryopreservation in a breeding program currently being conducted in

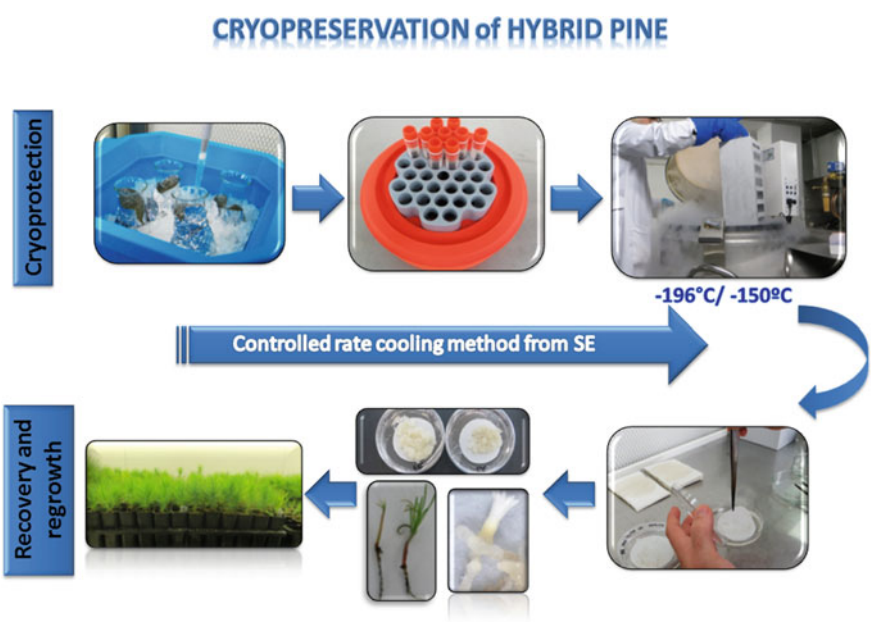

Fig. 2 Schematic diagram to cryopreserve and recovery *Pinus elliottii* × *P. caribaea* embryogenic cell lines, followed by the somatic plants regeneration

Brazil, new efforts are being implemented to increase the recovery rate of the ECL cryopreserved, in order to comprise all genotypes with a higher level of maturation rate. Moreover, the cryopreservation must be followed by a reliable protocol for regeneration via SE. Nunes et al. (2017b) described, for the first time, a complete protocol for PEE × PCH, from ECL induction to somatic plant regeneration. However, further improvements are needed to scale up, for the integration of the SE in the breeding programs of a MVF strategy. The initiation frequencies remain low and the maturation step is still dependent of the genotype.

For the successful integration of vegetative propagation within operational forestry, the genetic stability assessment of in vitro grown plants is an important requisite in SE protocols when coupled or not to cryopreservation, ensuring that regenerated plants are genetically identical to the donor material (Miguel and Marum 2011; Martinez-Montero and Harding 2015; Lelu-Walter et al. 2016). So, the plant viability and the genetic variability assessments of pine hybrid somatic plants recovered from EMs cryopreserved are important to validate the newly established cryopreservation protocols.

According to Nunes et al. (2017a), the cryopreservation procedure did not affect the PEE × PCH cryopreserved EMs recovery, the embryogenic capacity to mature embryos, the plant regeneration ability and no major genetic variations were also detected at ploidy DNA level. However, new studies need be performed to monitor

and analyze those plants in the field trials to evaluate the plant performance when reintroduced into natural environments (Martinez-Montero and Harding 2015).

A number of mechanisms for explaining the origin of putative somaclonal variation, have been already studied during in vitro culture and post-thaw storage, such as, different types of genomic perturbation, including numerical and structural chromosomal changes, point mutations, DNA methylation modifications and transposable element activations (Kaeppler et al. 2000; Miura et al. 2001; Peredo et al. 2008; Schellenbaum et al. 2008). However, during cryopreservation the occurrence of potential epigenetic changes can result from some reversible post-storage variability associated to storage stress adaptations (Martinez-Montero and Harding 2015). In conifers, the cryo-injury has been poorly addressed with only few cases (Park et al. 1998; Häggman et al. 1998; Krajňáková et al. 2011; Salaj et al. 2011). New studies should be performed to understand the linkage between the potential cryoinjury and stability in vitro.

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Oocarpa Pine (*Pinus Oocarpa* var. *Oocarpa* Schiede)

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1 Introduction

Pinus oocarpa var. *oocarpa* Schiede (Pinophyta, Pinales, Pinaceae subgenus Pinus) is found from southeastern Sonora, Mexico, to northern Nicaragua (Dvorak et al. 2000), and is known as the most common pine species in the southern half of Mexico and in Central America (Dvorak et al. 2009). It is primarily found in badly drained, non-fertile soil, and shallow, sandy, clay soils of moderate acidity (pH 4.0–6.5) (Dvorak et al. 2000).

Through provenance studies in South America, Africa and New Zealand, commercial plantations show some of the qualities of *P. oocarpa,* one of which is its excellent wood quality potential [Reviews by Dvorak et al. (2000) and Greaves (1982)]. Some uses for its wood include: lumber plywood, packing boxes, fuel wood, construction, broomstick handles, railroad ties, soft drink boxes, resin products, popsicle sticks, posts and kindling (Zamora 1981).

It hybridizes with a number of species (increasing the number of hybrids) (Dvorak et al. 2000) and has an average genome size of 21.74 pg/C (Hall et al. 2000), which is smaller than that of *P. radiata* (22.43 pg/C), but larger than that of *Pinus taeda* (21.27 pg/C) (Wakamiya et al. 1993). It is well adapted to nursery conditions; it is tolerant to moderate drought conditions and fire; and it is resistant to diseases by *Fusarium subglutinans* (Dvorak et al. 2000) and *F. circinatum*

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(Dvorak et al. 2009). The latter has received much attention because it is a serious problem in pine nurseries and plantations, especially for *P. radiata* and *P. patula* (Dvorak et al. 2009).

As stated above, this species has the potential for both commercial plantations and carbon sequestration (Alberto and Elvir 2008), along with its ability to hybridize with other pine species (Greaves 1982); however, little attention has been paid to the development of tissue culture protocols for its micropropagation (Schwarz et al. 1991).

Currently, clonal propagation methods are required to capture the genetic gains obtained through traditional breeding or the introduction of desirable genes via genetic modification into commercial varieties of tree species. Additionally, clonal propagation offers rapid and short-time production of reforestation stock, the production of woody biomass and the maintenance of elite and exotic germplasm (Giri et al. 2004). Thus, micropropagation is a prerequisite for low-cost and large-scale production of genetically improved germplasm of forest species; additionally, the price and efficiency of the production process for a specific species depends on the availability of protocols for efficient clonal propagation. Currently the most viable clonal propagation method is through the rooting of cuttings (Park et al. 2006).

In *P. oocarpa,* plantlet regeneration through clonal propagation has been reported via adventitious buds induced on explants obtained from 7- to 10-day old seedling tissues using a modified MS medium plus 0–25 nM NAA and 25 and 50 μM BA (Franco and Schwarz 1985), and from the formation and culture of calluses using protoplasts from 11-day old seedlings using 5 μM NAA and 10 μM zeatin (Laine et al. 1988). Thus, we outline here the clonal propagation through somatic embryogenesis for the first time using immature zygotic embryos.

2 Materials

2.1 Initiation and Capture of Somatic Embryogenesis

1. Plant material: *Pinus oocarpa* cones (containing zygotic embryos at the pre-cleavage stage, the cleavage stage and late cotyledonary stages), forceps, scalpel and zoom stereomicroscope.
2. Cone disinfection: soap, 70% (v/v) ethanol, 2.5% (v/v) commercial bleach (sodium hypochlorite) solution, Tween-20 and sterile deionized water.
3. Culture media: Standard methods are used to prepare the macroelements of each medium (Table 1); a medium pH is adjusted with KOH or HCl. Phytagel is added to 0.3% (w/v) prior to autoclaving at 121 °C for 25 min. The microelements, carbohydrate source, vitamins, amino acids (Table 1) and plant growth regulators (Table 2) are filter sterilized, and added after autoclaving to the macroelements, which have been cooled to ~55 °C. Pour 25 ml per petri dish.

Table 1 Concentrations of salts and other components used for the initiation of somatic embryogenesis in *P. oocarpa*

Formula	Concentration in basal media (mg/L)	
	Induction and capture	Maturation
	1250 (Pullman et al. 2006)	927 (Pullman et al. 2003)
KNO_3		454.90
$Ca(NO_3)_2 \cdot 4H_2O$	236.00	59.10
NH_4NO_3	603.80	200.00
KH_2PO_4		136.10
Mg(NO3)2 · $6H_2O$		256.50
$MgCl_2 \cdot 6H_2O$		101.70
$MgSO_4 \cdot 7H_2O$		246.50
$FeSO_4 \cdot 7H_2O$	6.95	41.70
$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$	9.33	55.95
KI		4.15
H_3BO_3		7.75
$ZnSO_4 \cdot 7H_2O$		14.40
$MnSO_4 \cdot H_2O$		10.50
$Na_2MoO_4 \cdot 2H_2O$		0.13
$CuSO_4 \cdot 5H_2O$		0.13
$CoCl_2 \cdot 6H_2O$		0.13
Biotin	0.05	
Folic acid	0.50	
Thiamine HCl (B1)		1.00
Nicotinic acid		0.50
Pyridoxine HCl (B6)		0.50
Glycine		2.00
PEG 8000		130,000.00
Myo-inositol	10,000.00	100.00
Maltose		20,000
Casamino acids		500.00
L-glutamine		450.00
MES		250.00
Sucrose	30,000.00	

4. Double staining of tissues: 2% (w/v) acetocarmine, microcope slides, gas burner, deionized water, 0.5% (w/v) Evan's blue, glycerol and zoom stereomicroscope.
5. Tissue culture chamber set at 25 °C.
6. Captured cultures are transferred to the same induction medium.

Table 2 Plant growth regulators for somatic embryogenesis of *Pinus oocarpa*

Plant growth regulators	Concentration (μM)		
	Induction and capture	Multiplication	Maturation
NAA	–	–	
2,4-D	10.0	2.5	
BA	4.0	1.0	
Kin	4.0	1.0	
ABA	10.0	2.5	40

2.2 Multiplication and Maturation of Somatic Embryos

1. Multiplication: Petri dishes containing the basal medium 1250 (Table 1; Pullman et al. 2006) plus plant growth regulator concentrations (Table 2).
2. Maturation: Filter paper and petri dishes containing the basal medium. The basal maturation medium is the 927 medium (Pullman et al. 2003) plus 6% (w/v) maltose, 12% (w/v) PEG 8000, 0.6% (w/v) Phytagel combined and 40 μM ABA.

2.3 Germination of Mature Somatic Embryos

1. Six-well plates, sterile water and a growth chamber set at 25 °C.
2. GA-7 vessels containing autoclaved peatmoss:vermiculite (3:1).
3. A liquid germination medium. The basal germination medium consists of ½ MS medium plus 0.25 mg/L copper sulfate, 100.0 mg/L myo-inositol, 0.3% (w/v) Phytagel, 0.25% (w/v) activated charcoal and 2% (w/v) maltose.

3 Methods

3.1 Initiation of Somatic Embryogenesis from Immature Zygotic Embryos

Cones Containing Zygotic Embryos at the Late Cotyledonary Stage:

4. Extract seeds from cones containing embryos at the late cotyledonary stage and place them into a petri plate at 4 °C until disinfection.
5. For disinfection, place seeds inside a tea ball and rinse them with tap water for 3 min.

6. Place the tea ball inside a beaker containing 70% (v/v) ethanol for 2 min.
7. Rinse once with sterile, deionized water.
8. Disinfect seeds by soaking them, while stirring, in a 5% (v/v) commercial bleach (sodium hypochlorite) solution supplemented with a drop of Tween-20 for 15 min.
9. Rinse four times (2 min each) with sterile deionized water.
10. Remove the seed coat and dissect the megagametophyte out.
11. Partially remove the zygotic embryo, allowing the embryo to remain connected to the tissue.
12. Place the embryos with the megagametophyte facing down on the media.
13. Incubate the embryos in the tissue culture chamber in the dark.

Cones Containing Immature Zygotic Embryos (pre-cleavage and pre-cotyledonary stages):

1. Gently wash cones with soap and water.
2. Disinfect the cones in 70% (v/v) ethanol for 1–2 min.
3. Rinse once with sterile deionized water.
4. Add 2.5% (v/v) commercial bleach (sodium hypochlorite) solution supplemented with 5 drops of Tween-20 per 100 ml of solution for 5 min.
5. Rinse three times in sterile deionized water for 2 min.
6. Extract the seeds and dissect the megametophytes out.
7. Place the intact megagametophytes onto initiation media.
8. Incubate the megagametophytes in the tissue culture chamber in the dark.

Double Staining of Tissues:

Extrusions appear after four to six weeks in culture (Fig. 1a). Once the somatic embryogenic tissue is captured and exhibits continued growth, a portion of the tissue is used to confirm the embryogenic state (Fig. 1b). Embryogenic tissue is identified by its morphological characteristics, including a translucent to white mucilaginous appearance of the tissue, and through histological analysis (Gupta and Holmstrom 2005; Fig. 1c).

1. Briefly, place the tissue on a glass slide and carry out the first stain with 2% (w/v) acetocarmine, making sure that the tissue is completely submerged.
2. Tease the tissue apart to make sure it is accessible to the stain, and heat the slide over a low flame or heat source for a few seconds.
3. Wash the tissue twice with deionized water.
4. Add the second stain, 0.5% (w/v) Evan's blue, and after 30 s wash twice with water.
5. Mount the tissue into glycerol to prevent drying, and overlay with a cover slip.
6. Make observations under a zoom stereomicroscope.

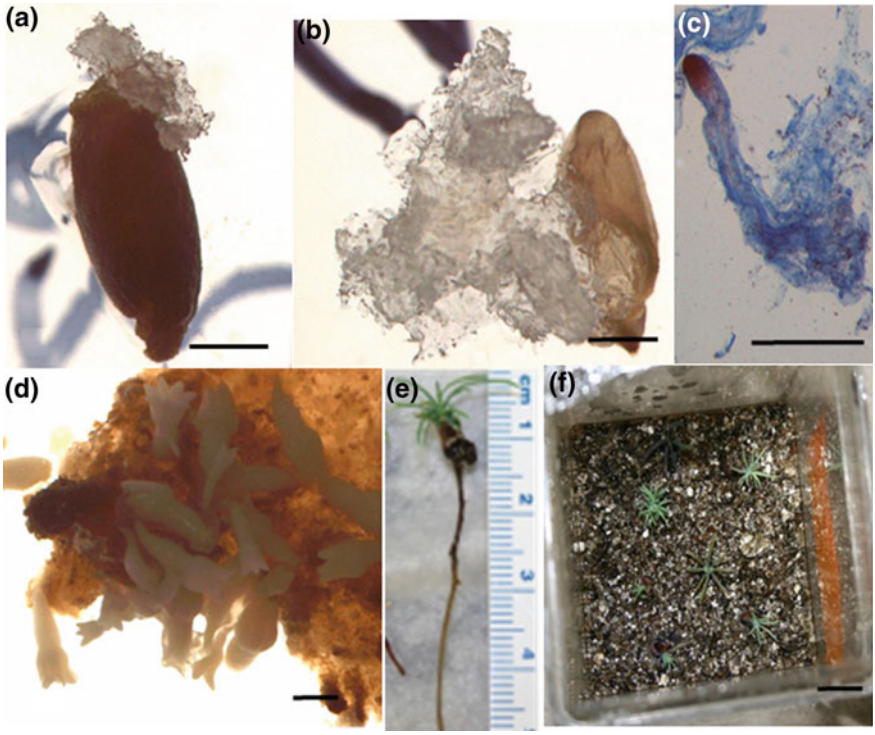

Fig. 1 Plant regeneration of *Pinus oocarpa* through somatic embryogenesis: Somatic embryo tissue: **a** 6 weeks. **b** 8 weeks on initiation medium. **c** Double staining of embryogenic suspensor mass (ESM) showing single embryo. **d** Maturation of somatic embryos; (bar = 1 mm). **e** Germination of somatic embryos using desiccation treatments under the hood. **f** Somatic seedlings in GA-7 vessels before transfer to ex vitro conditions (bar = 0.5 cm)

3.2 Multiplication of Embryogenic Suspensor Mass

After three months from the onset of induction, cell lines originating from explants are placed on the multiplication medium. It has been found that during the multiplication of conifer somatic embryos, concentrations of 2,4-D and BA are usually reduced or are kept at the same concentrations used for initiation (Häggman et al. 2006).

1. Place a small amount of embryogenic suspensor mass (ESM) onto a petri plate containing the multiplication medium.
2. Place cultures in the tissue culture chamber in the dark.
3. Perform subcultures by transferring ESM onto a fresh medium at ten-day intervals to increase the embryogenic tissues.

3.3 Maturation of Somatic Embryos

ABA has been demonstrated as essential in the further development of the embryos; however, the concentration required is both species and genotype dependent. Based on previous results from our laboratory with *P. taeda* showing that 40 μM ABA yielded better embryo morphology and germination, the same concentration is used for the maturation of somatic embryos of *P. oocarpa* (Fig. 1d). Additionally, polyethylene glycol (PEG) is a high molecular weight, non-penetrating osmotic agent, which can be added to the medium to create an osmoticum, and which exerts water stress similar to natural drying (Attree and Fowke 1993).

1. Place the ESM on a filter paper (Whatman #4) and into a petri dish containing the maturation medium.
2. Place cultures in the tissue culture chamber in the dark.
3. Subculture the tissue by transferring it to a fresh medium every five weeks.
4. After 2 weeks on the maturation medium, embryos with small yellow heads are observed, which enlarge and develop into fully mature embryos after 10 weeks in culture. Embryos progress through round-globular, then early and late cotyledonary developmental stages.

Germination of Mature Embryos:

Germination success is considered as the elongation of both the root and shoot (Fig. 1e). It is achieved by giving the mature somatic embryos a desiccation treatment to mimic the natural conditions of the seeds (Roberts et al. 1990); and by providing a germination medium which consists of a plant growth regulator-free medium with a low salt concentration, and the addition of activated charcoal to absorb any growth regulators carried over from maturation.

1. Place five mature embryos into each of three separate wells of a six-well plate, with the remaining three wells filled with 3 ml of sterile water [high humidity desiccation (HHD)] (Roberts et al. 1990).
2. Seal the plates and place them in the tissue culture chamber in the dark.
3. After 2–3 weeks, transfer the embryos to the germination medium, culture them in the dark one more week and then place them under a cool white fluorescent light at an intensity of 75.71 μmol m^{-2} s^{-1}, at 25 °C and a photoperiod of 16/8-h (day/night).
4. Root elongation is observed after 7 days following embryo transfer to light. Elongation of both the root and shoot is observed after 14 days, and at 28 days, synchronized root and shoot elongation is observed.
5. Transfer the somatic seedlings to GA-7 vessels containing autoclaved peatmoss: vermiculite (3:1) moistened with the liquid germination medium.
6. Transfer seedlings to ex vitro conditions after 4 weeks (Fig. 1f).

4 Improvements to the Protocol

To our knowledge, this is the first report on the production of somatic seedlings in *P. oocarpa* through somatic embryogenesis (Fig. 1; Lara-Chavez et al. 2011). Furthermore, the gene expression analysis of six molecular markers: Legumin-like, Vicilin-like, group 4 late embryogenesis abundant (LEA), homeodomain-leucine zipper I (HD-Zip I), 26 S proteasome regulatory subunit S2 (RPN1) and Clavata-like) were studied through embryo development. Results showed that even though all six markers were expressed during both zygotic and somatic embryogenesis, improvements are needed for the optimization of somatic embryo development (Lara-Chavez et al. 2012). Therefore, improvements such as the following are needed:

(1) Somatic embryogenesis is best supported by mimicking natural seed–embryo developmental conditions, through a tissue culture medium formulation based on the mineral content of the seed nutritive tissue, megagametophyte. An attempt to develop a novel culture medium (*P. oocarpa* medium—PO) was tested in combination with different plant growth regulator concentrations. Although the PO medium performed suboptimally for the induction of the somatic embryos, on specific cell lines, it did show the potential for enhancing both the culture proliferation and embryo maturation. This was supported by a microscopic analysis in which better embryo morphology was observed on the PO medium compared with embryos on standard *Pinus taeda* media.
(2) Multiplication and proliferation of growth of the *P. oocarpa* cell lines could be improve by suspending and dispersing the cell clumps of tissue onto filter paper discs. This methodology has been found to substantially increase embryogenic line proliferation for other pines species (Klimaszewska and Smith 1997; Aronen et al. 2009; Carneros et al. 2009).
(3) While maturation was obtained on 40 μM ABA, further studies on the effects of a broader range of ABA concentrations would lead to enhanced somatic embryo maturation percentages. In addition, to test the effects of gelling agents, carbon sources, anti-auxins and their concentrations (Carneros et al. 2009) may serve to enhance the process.

5 Future Opportunities

A variety of unique opportunities exist for future work involving *Pinus oocarpa* somatic embryogenesis. While somatic embryogenesis protocols allow for large scale clonal propagation and the deployment of select germplasm, widespread use still depends on a confirmation of somatic seedling quality and performance. Ecophysiological and performance studies have been carried out with spruce somatic seedlings, and have shown that high morpho-physiological quality

seedlings in a forest nursery can be obtained from crosses between somatic clones (Colas and Lamhamedi 2014), and that somatic and zygotic seedlings are highly comparable in their field performance (Grossnickle and Major 1994). However, at present, large-scale nursery and field performance trials, including these types of ecophysiological studies, remain to be performed with somatic seedlings of Oocarpa pine.

Apart from the propagation of superior germplasm, somatic embryogenesis provides for the rapid development of improved, modified materials through the use of genetic engineering technologies. The adoption of these technologies requires a viable and functional transformation system coupled with a robust, somatic embryogenesis regeneration system. Transformation protocols for various pines have been developed using both organogenesis (Tang 2003; Tang et al. 2014) and somatic embryogenesis (Wenck et al. 1999; Tang et al. 2001; Alvarez and Ordás 2013; Le-Feuvre et al. 2013) regeneration systems. While there are no reports of Oocarpa pine transformation as of yet, the preliminary work of identifying *Agrobacterium* strains that work well with pine species, developing efficient plasmid constructs and protocols for pine transformation provide excellent starting points for future research targeting *P. oocarpa* transformation using somatic embryogenesis as the preferred regeneration route.

Genetic engineering technologies offer the potential for novel trait development through gene/allele modifications that might not occur naturally, while also allowing trait development to occur more rapidly than through traditional breeding approaches (Osakabe et al. 2016). A variety of traits can be targeted for improvement, including growth, wood characteristics and composition, and disease resistance. However, genetic engineering requires some knowledge of the target genes/gene sequences to be modified for trait improvement. Advances in sequencing technologies over the last several years have allowed whole genome sequences of industrially-important woody plant species to be obtained (Osakabe et al. 2016), including loblolly pine (Neale et al. 2014; Zimin et al. 2014) and sugar pine (Stevens et al. 2016). The availability of various pine whole genome sequences and genotyping by markers provides the ability to begin to identify the genes associated with specific traits and QTLs, and also the potential to begin gene and allele discovery in Oocarpa pine, providing candidate genes for trait modification via transgenic technologies. The exciting recent developments in genome editing using the CRISPR/Cas9 and CRISPR/CpfI systems (Tsai and Xue 2015; Puchta 2017; Tang et al. 2017) coupled with whole genome sequencing and robust transformation technologies using somatic embryogenesis regeneration, offers the potential for targeted gene modifications with various forestry species, including Oocarpa pine.

As indicated above, numerous advances in ecophysiological characterization, transformation technologies, genome sequencing, gene discovery, and genome editing, coupled with advancements and improvements in *Pinus oocarpa* somatic embryogenesis protocols, provide a wealth of tools that can be applied to further development of this important southern pine species.

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Somatic Embryogenesis in Siberian Dwarf Pine (*Pinus pumila* (Pall.) Regel)

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1 Introduction

Siberian dwarf pine (*Pinus pumila* (Pall.) Regel) belongs to the five-needle pines, grows in northeastern Siberia, and occupies a vast area extending from the northern Siberia to the Kamchatka Peninsula. This species has of great ecological and economic value. Its thickets are widely used for landscape. *P. pumila* seeds serve as an important food source for animals. However, this species is seriously threatened in the larger area due to long-term forest fires, which hamper its natural regeneration.

The problems of restoration of the *P. pumila* population and its gene pool conservation can be solved by micropropagation through somatic embryogenesis. This method is useful to perform mass reproduction of this species with traits valuable for breeding purposes. Various techniques including DNA microarrays, genetic transformation, and cryopreservation of somatic embryogenic lines to establish a germplasm bank of valuable genotypes, have been used. The somatic embryogenesis of *P. pumila* can serve for both practical purposes and as a model system for studying the patterns of cell differentiation and realization of the morphogenetic developmental program in early ontogeny (Lelu et al. 1994; von Arnold et al. 2002; Park and Lelu-Walter 2006).

To date, embryogenic callus and somatic embryos have been obtained for 28 species of the genus *Pinus* (Gupta and Dursan 1987; Laine and David 1990; Salajova et al. 1995; Garin et al. 1998; Lelu et al. 1999; Arya et al. 2000; Percy et al. 2000; Klimaszewska et al. 2001; Niskanen et al. 2004; Lelu-Walter et al. 2008; Pullman and Bucalo 2011; Tretyakova and Voroshilova 2014; Tretyakova and Shuvaev 2015). Mostly the culture conditions were optimized ensuring the plant regeneration from somatic embryos.

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This chapter describes the proliferation of somatic embryogenic cell lines of *P. pumila.* This protocol is based on somatic embryogenesis initiated from immature zygotic seeds and plant regeneration obtained from somatic embryos after maturation with polyethylene glycol. Procedures including explant preparation, embryonal mass induction and proliferation, somatic embryo maturation, germination, and plantlets acclimatization are described.

2 Protocol for Somatic Embryogenesis in Siberian Dwarf Pine

2.1 Culture Media

1. The culture media ½LV1 (Litvay et al. 1985) used for Siberian draft pine somatic embryogenesis described in Table 1.
2. Note that this protocol consists of several in vitro culture stages differing in medium, culture condition, and duration of culturing as described in Table 2.
3. Adjust medium to pH 5.8, and autoclave for 15 min at 121 °C and 1.1 kg cm^{-2}.
4. Ascorbate acids and abscisic acid (ABA) are filter sterilized and added to the medium after autoclaving.

2.2 Explants Preparation

1. Collection of immature cones from mother trees in middle July (Fig. 1).
2. Removing the seeds from the immature cones.
3. Megagametophytes with immature zygotic embryos at the globular stage were used as explants.
4. The planting material was sterilized in two steps. The first step included the surface treatment of seeds with 0.3% potassium permanganate for 5 min, followed by seed washing 3 times with sterile distilled water, 3 min each time. Megagametophytes with immature zygotic embryos without the seed coats were sterilized in a 3% alcohol in iodine solution (3 g iodine dissolved in 95% alcohol, density of alcohol 0.8) for 5 min. They were washed 3 times with sterile distilled water, 3 min each time.
5. Megagamethophytes with immature zygotic embryos were cultured on the surface of ½LV1 agar nutrient medium for initiation.

Table 1 Constituents of culture media for Siberian dwarf pine regeneration system via somatic embryogenesis

Constituents	LV1 (mg/L)	LV2 (mg/L)	LV3 (mg/L)	LV4 (mg/L)	LV5 (mg/L)
Basal salts					
KNO_3	950	950	950	950	950
$NaNO_3$	825	825	825	825	825
KH_2PO_4	170	170	170	170	170
$MgSO_4{\cdot}7H_2O$	925	925	925	925	925
$CaCl_2{\cdot}2H_2O$	11	11	11	11	11
$MnSO_4{\cdot}4\ H_2O$	31	31	31	31	31
H_3BO_3	4.15	4.15	4.15	4.15	4.15
$ZnSO_4{\cdot}7\ H_2O$	27.7	27.7	27.7	27.7	27.7
$CuSO_4{\cdot}5\ H_2O$	1.25	1.25	1.25	1.25	1.25
$Na_2MoO_4{\cdot}2\ H_2O$	43	43	43	43	43
$CoCl_2{\cdot}6\ H_2O$	0.5	0.5	0.5	0.5	0.5
$FeSO_4{\cdot}7\ H_2O$	27.8	27.8	27.8	27.8	27.8
Na_2-EDTA	37.3	37.3	37.3	37.3	37.3
Vitamins					
Myo-Inositol	1000	1000	1000	1000	
Thiamine hydrochloride	0.1	0.1	0.1	0.1	
Pyridoxine hydrochloride	0.5	0.5	0.5	0.5	
Nicotinic acid	0.5	0.5	0.5	0.5	
Plant growth regulators					
2,4-D	2.0	2.0			
BAP	1.0	0.5			
ABA				32	
IBA				0.2	0.2
Other additives					
Casein hydrolysate	500	500	500	500	
Glutamine	500	500	500	500	
Ascorbic acid	500	300	300	300	
Sucrose	50,000	20,000	30,000	30,000	
Polyethylene glycol				100,000	
Gellan gum				4000	4000
Agar	7000	7000			
pH	5.8	5.8	5.8	5.8	5.8

LV1 (Embryogenic culture initiation medium), LV2 (Embryonal mass maintenance/proliferation medium), LV3 (Somatic embryo pre-maturation liquid medium) (suspension culture, on a rotary shaker, 60 rpm), LV4 (Somatic embryo maturation medium), LV5 (Somatic plant germination medium)

Table 2 Medium, culture conditions, and culture durations for each stage of somatic embryogenesis in Siberian draft pine

Stage	Medium[a]	Culture conditions[b]	Duration (week)
1. Embryogenic culture initiation	LV1	Dark, 90 × 15 mm Petri dishes (30–35 ml medium/plate) 4 megagamethophytes with embryos/plate	4–12
2. Embryonal mass maintenance/ proliferation	LV2	Dark, 90 × 15 mm Petri dishes (30–35 ml medium/plate) 4 embryonal masses/plate	2–12
3. Somatic embryo prematuration	LV3	Dark, 90 × 15 mm Petri dishes 30–35 ml medium/plate 4 embryonal masses plate	1
4. Somatic embryo maturation	LV4	Dark, 90 × 15 mm Petri dishes 35–40 ml medium/plate embryos'	11–12
5. Somatic embryo germination	LV5	Light, 90 × 15 mm Petri dishes (100 ml medium/plate)	6–8
6. In vitro growth of somatic plants		Light, 200 ml Vassals. Subs tract: sand: vermiculite: peat (1:1:1)	82

[a]See Table 1
[b]Culture at 16-h photoperiod (65 μmol m^{-2} s^{-1}) or darkness at 24 ± 1 °C

Fig. 1 Immature cones of *Pinus pumila i*n July. Bar: 1 cm

2.3 *Embryogenic Culture Initiation*

1. For the induction of embryonal masses, the megagametophyte with immature zygotic embryos were put horizontally on the surface of the initiation medium (LV1, Table 1) in Petri dishes (four explants per plate).
2. The presence of early stages of embryogenic callus was observed on LV1 after 6 week cultivation (Fig. 2). During this time, the first subculture were conducted on LV2.

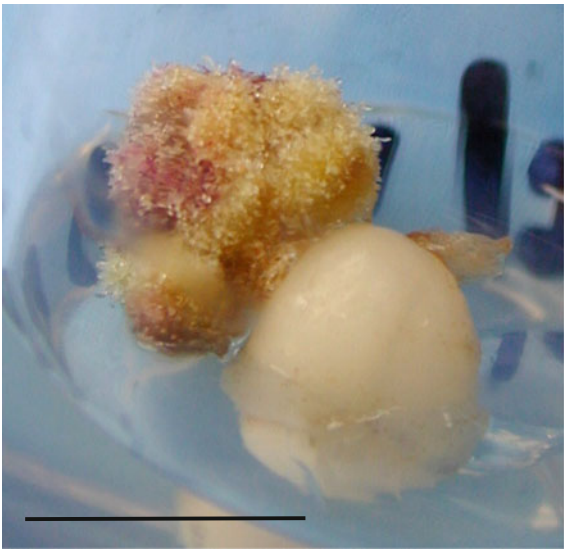

Fig. 2 Induction of embryonal mass from megagametophyte explant of *Pinus pumila* in the medium LV1. Bar: 1 cm

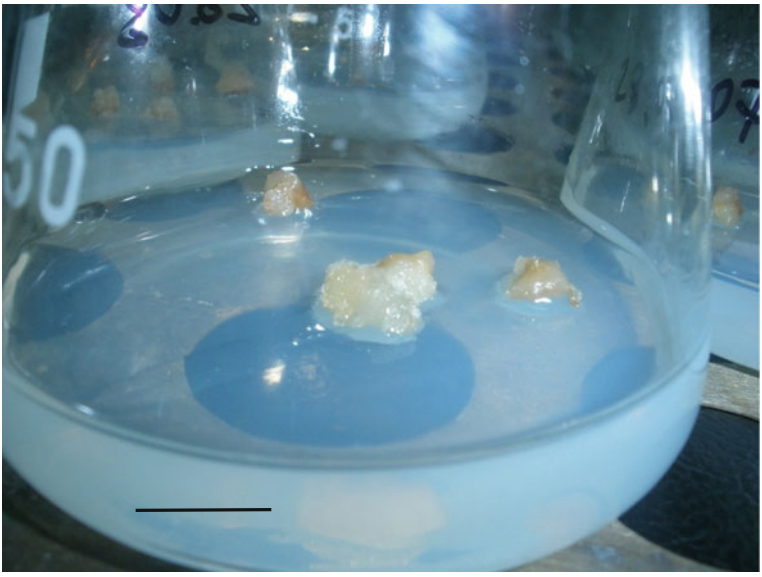

Fig. 3 Embryonal mass in proliferation medium after first 2 week of subculture on LV2. Bar: 1 cm

2.4 Maintenance and Proliferation of Embryonal Masses

1. Embryonal masses from initiation medium LV1 were collected and transferred to maintenance/proliferation medium (LV2, Table 1).
2. After 2–3 week of culturing on LV2 medium embryonal mass formed (Fig. 3).
3. Embryonal masses were developed in 10 weeks on the LV2 media with regular subculture at 2–3 week interval into the fresh medium (Fig. 4).
4. For the histological study squashed preparations were used. After 4-10 week of culturing on the LV2 medium, small piece of ESM was placed onto glass slide and kept in 1% safranin solution or 1% carmine solution (Tretyakova 2013). The embryonal head cell stained red and suspensor system (elongated cells) stained less bright (Fig. 5).
5. Before maturation, transfer 50 mg proliferated embryonal mass to 100 ml flasks containing hormone—free 50 ml liquid LV3 medium. The suspension cultures were grown in the darkness on a rotary shaker, shaking at 60 rpm (Table 2).
6. Histological study after 1 week on the proliferation LV3 medium, showed that the embryogenic cultures consist of globules and suspensor cells (Fig. 6).

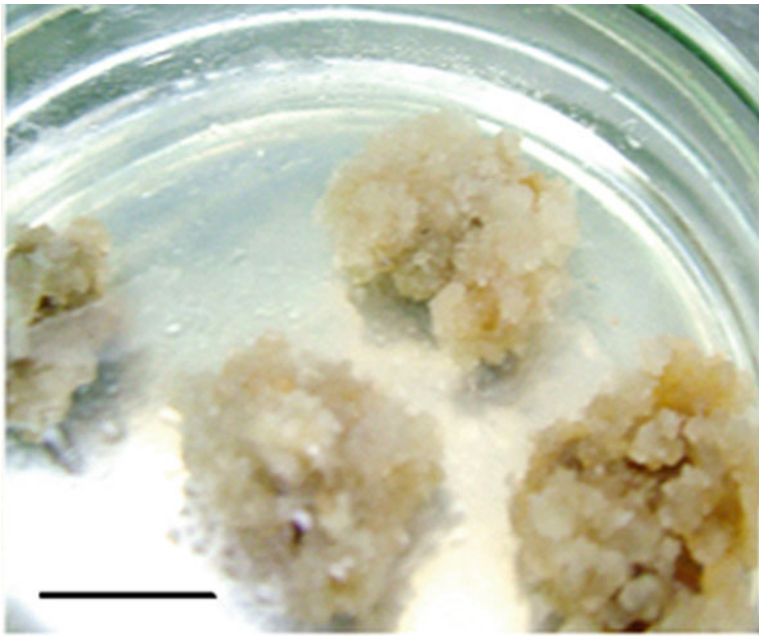

Fig. 4 Embryogenic cultures during 10 week on the LV2 medium. Bar: 1 cm

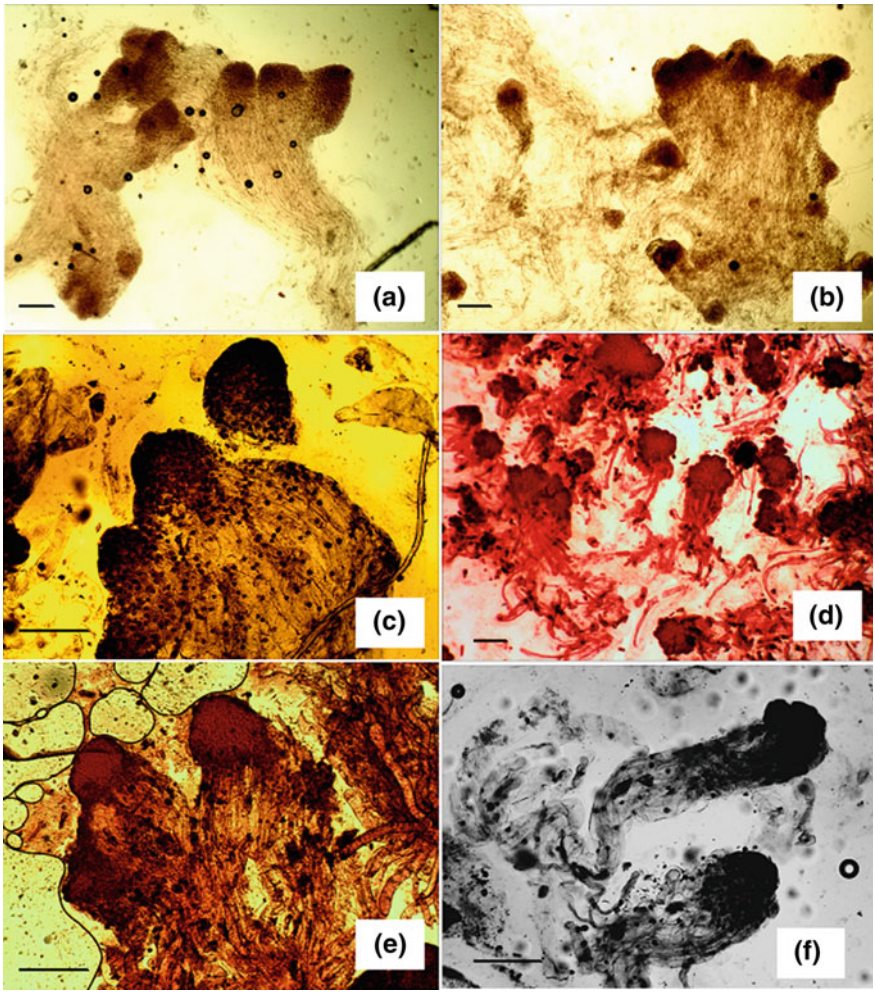

Fig. 5 Embryonal-suspensor mass during 4–10 week culturing on the media LV2: **a–d** polyembryonic complexes consisting of several embryos; **e** the multiplication of somatic embryos via cleavage; **f** separate somatic embryos. Bar 200 mkm

2.5 Maturation of Somatic Embryos

1. Maturated somatic embryos are transferred to LV4 containing 4000 mg/l gellan gum, 100,000 mg/l polyethylene glycol, 0, 2 mg/l IBA and 32 mg/l ABA. The ESM was transferred to the solidified LV4 medium on sterile filter paper (diameter 50 mm), placed in a Bucher funnel (upper diameter 60 mm, lower diameter 15 mm, pore diameter 1, 25 mm). The suspension (25 ml) was poured into the funnel and the liquid fraction was removed via OM-1 medical aspirator.

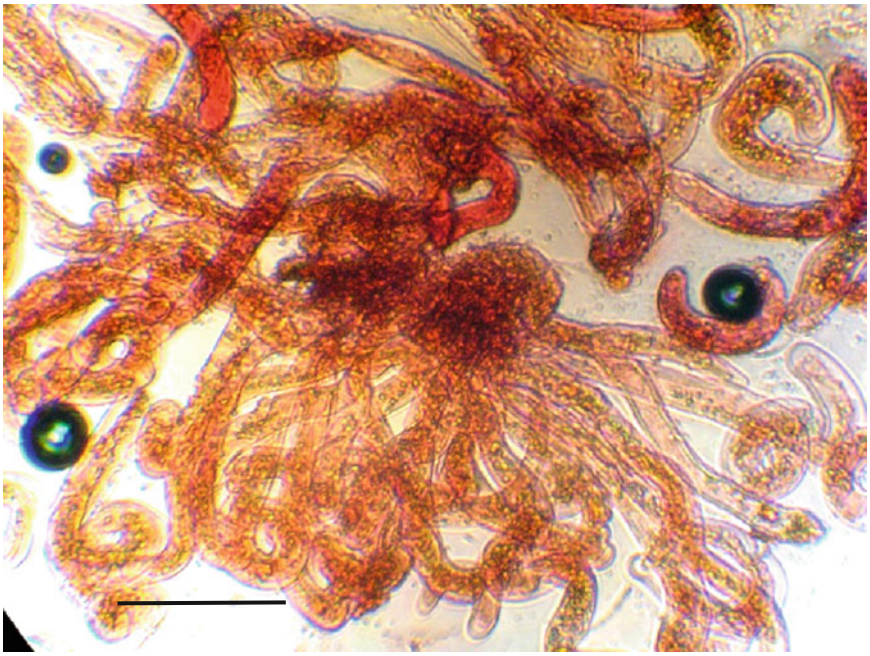

Fig. 6 Embryonal-suspensor mases after 1 week culturing on medium LV3. Bar 50 mkm

After filtration, the filter paper with the ESM residue was transferred onto the surface of the LV4 nutrient medium for maturation.

2. Somatic embryos on LV4 medium formed after 40 days of culturing. From 1 g of ESM developed 36–63 embryos (Fig. 7).

2.6 Germination and Acclimatization of Somatic Embryos

1. Collect filter paper disks containing somatic embryos and transfer onto the plates containing embryo germination medium for 4–8 weeks (medium LV5, Table 1; Fig. 8a).
2. For acclimatization the morphologically normal plantlets with both shoots and roots that develop from somatic embryos are transferred to vassals containing soil (sand, vermiculite and peat in a ratio 1:1:1) (Fig. 8b, c; Table 2).

Fig. 7 Maturation somatic embryos on medium LV4 (development of cotyledons, hypocotyls and root meristems are shown by arrows). Bar: 1 cm

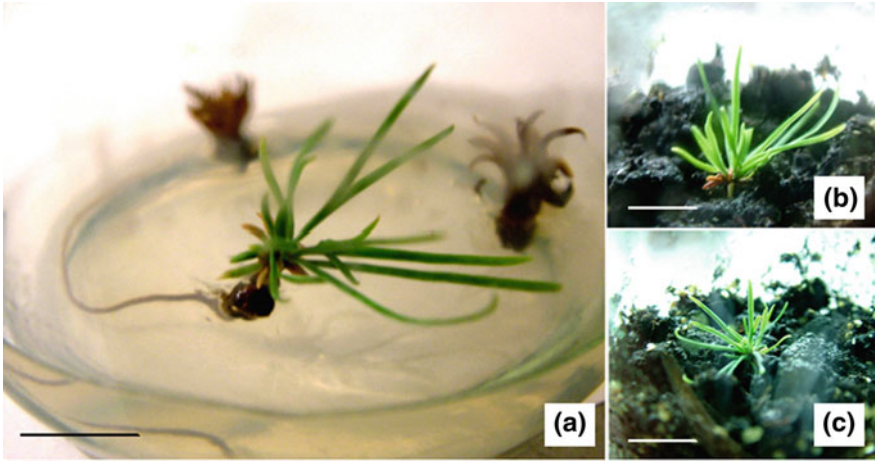

Fig. 8 Regenerants of *Pinus pumila*: **a** on media LV5; **b**, **c** soil in vitro: sand, vermiculite and peat). Bar: 1 cm

3 Conclusion and Future Prospects

The somatic embryogenesis in Siberian draft pine has been initiated in vitro cultures by culturing megagametophytes having immature zygotic embryos at the globular developmental stage were placed on the medium 1/2 LV (Table 1). Embryogenic cell cultures were multiplied as a result of somatic polyembryogenesis via cleavage. The embryogenic productivity was high. After maturation on the medium containing polyethylene glycol, mature somatic embryos germinated and regenerated plants. This is the first study showing induction of the embryogenic cultures, and somatic embryo formation in *Pinus pumila*. These results will contribute to the formation of high-productivity nut plantations.

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