PROTOCOL FOR SOMATIC EMBRYOGENESIS IN WOODY PLANTS

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Protocol for Somatic Embryogenesis in Woody Plants

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S. Mohan Jain

and

Pramod K. Gupta

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Preface

World population is increasing at an alarming rate and this has resulted in increasing tremendously the demand for tree products such as wood for construction materials, fuel and paper, fruits, oils and medicines etc. This has put immense pressure on the world's supplies of trees and raw material to industry and will continue to do so as long as human population continues to grow. Also, the quality of human diet, especially nutritional components, is adversely affected due to limited genetic improvement of most of fruit trees. Thus there is an immediate need to increase productivity of trees. Improvement has been made through conventional breeding methods, however, conventional breeding is very 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. In 1958, Reinert in Germany and Steward in USA independently reported somatic embryogenesis in carrot cultures. Since then, tremendous progress in somatic embryogenesis of woody and non-woody plants has taken place. 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. These provided readers detailed reviews on somatic embryogenesis of important angiosperm and gymnosperm tree species, which included extensive review of literature. This provided an excellent source of information for new comers and people already engaged in research. However, these book volumes did not provide "detailed protocols" for inducing somatic embryogenesis. As a result, there may be difficulties in initiating somatic embryogenesis cultures (*e.g.* the choice of explant is one of the important parameters could affect initiation of embryogenic cultures). This book provides chapters on stepwise protocols of somatic embryogenesis of a range of selected woody plants, so that researchers can initiate somatic embryogenic cultures without too much alteration.

This book has a total of 46 chapters; and divided into four sections A, B, C, and D.

Section A has 12 chapters on conifers included are: Slash pine (*Pinus elliottii*), radiata pine *(Pinus radiata),* Douglas–fir *(Pseudotsuga menziesii*), Omorika spruce

(Picea omorika), white spruce (*Picea glauca)*, Black spruce (*Picea mariana),* Sitka spruce *(Picea sitchensis),* Black pine (*Pinus nigra)*, Loblolly pine *(Pinus taeda)*, maritime pine *(Pinus pinaster),* Mexican weeping pine (*Pinus patula)*, Norway spruce (*Picea abies*)

Section B contains 14 chapters on fruits, which are: Cashew (*Anacardium occidentale),* coffee (*Coffea arabica* and *C.canephora)*, cacao (*Theobroma cacao)*, mango *(Mangifera indica)*, jackfruit *(Artocarpus heterophyllus),* Indian olive *(Elaeocarpus robustus),* bottle palm (*Hyophorbe lagenicaulis*), American grapes *(Vitis x labruscana),* pistachio *(Pistachio vera),* grapes *(Vitis vinifera)*, date palm (*Phoenix dactylifera),* tea *(Camellia sinensis),* citrus (*Citrus* spp.*),* and olive *(Olea europaea)*.

Section C deals with 14 chapters on Indian rosewood (*Dalbergia sissoo)*, Pedunculate oak *(Quercus robur),* sessile oak *(Quercus petraea),* tamarillo (*Cyphomandra betacea),* European chestnut *(Castanea sativa),* Babul *(Acacia arabica)*, hazelnut (*Corylus avellana*), Canela-preta (*Ocotea catharinensis)*, cork oak (*Quercus suber),* Sawara cypress (*Chamaecyparis pisifera),* Holm oak (*Quercus ilex)*, hybrid firs (*Abies alba x A. cephalonica*), sandalwood (*Santalum album*), Purple caneflower (*Echinacea purpurea)*

Section D includes 6 chapters on histological studies, bioencapsulation, protoplast isolation and culture, cryopreservation, double staining technology, and thin cell layer sectioning.

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.* protoplasts, encapsulation, cryopreservation, genetic transformation, genetic fidelity with molecular markers, and bioreactor.

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 book chapters that have maintained high quality of the book.

S. Mohan Jain
Pramod Gupta

SLASH PINE (*PINUS ELLIOTTII* Engelm.)

Ronald J. Newton[1], Wei Tang[1], and S. Mohan Jain[2]

[1]East Carolina University, Greenville, NC 27858-4353, USA
[2]International Atomic Energy Agency, FAO/IAEA Joint Division, Vienna, Austria

1. INTRODUCTION

Slash pine (*Pinus elliottii* Engelm.) is one of the most important tree species in the southern pine region. Millions of acres of slash pine have been planted, grown and harvested because of its fast growth and excellent utility for pulp, lumber, poles, and gum naval stores. Slash pine has successfully been grown in plantations in Africa, China, South America, and Australia. In those areas where slash pine is concentrated, thousands of jobs and millions of dollar industries depend on this species. It takes about 30 years for slash pine trees to reach saw-timber size. Younger trees are harvested for pulpwood that is converted to many products in paper industries. Slash pine is also used to produce turpentine and rosins for many chemical industries. It is identified with large, flat bark plates, rough twigs, and large brooms of needles. Needles are 5 to 11 inches long and are packaged two or three per fascicle. Slash pine occurs naturally in wet, flat-woods, swampy areas, and shallow pond edges. Slash pine is sometimes found growing with loblolly pine (*Pinus taeda* L.) The two species can be distinguished by noting that loblolly pine always grows with three needles per fascicle, its cones persist on the tree for a longer time, and cones of loblolly pine are far more prick, than slash pine cones.

Slash pine is one of the fastest growing and most commercially important yellow pines in the tropical and subtropical regions in the world. Two varieties of this pine are recognized: *Pinus elliottii* var. *elliottii*, slash pine, which grows naturally throughout the lower southeastern states in U.S., and *Pinus elliottii* var. *densa*, South Florida slash pine, which occurs only in the southern half and Keys of Florida. This later variety differs from the typical variety not only in geographical location, but also in seedling development and wood density. South Florida slash pine produces denser wood.

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 1–10.

Somatic embryogenesis is an important and useful technique in (1) large-scale propagation of superior and genetically engineered forest tree, (2) the production of synthetic seed, and (3) as a target for genetic engineering (Attree and Fowke, 1993; Gupta and Durzan, 1991; Jain et al. 1995; Gupta et al. 1988; Wenck et al. 1999). Somatic embryogenesis in conifers was first described relatively by three independent groups (Hakman and von Arnold 1985; Chalupa 1985; Nagmani and Bonga 1985). It has been demonstrated that mature conifer somatic embryos produced via plant tissue culture techniques appear to have the same quality as mature zygotic embryos; therefore, somatic embryogenesis is a potential choice and resource for the study of embryo developmental physiology and biotechnological applications in clonal forestry (Attree and Fowke, 1993; Jain et al. 1995).

In vitro regeneration of slash pine has proven to be very difficult. There are only a few reports on plant regeneration via somatic embryogenesis slash pine. Jain et al. (1989) first reported somatic embryogenesis and plant regeneration from immature zygotic embryos cultured on DCR medium (Gupta and Durzan 1986). Newton et al (1995) reported somatic embryogenesis, gene transfer, and transient expression of β-glucuronidase gene (*GUS*) in slash pine (Jain et al. 1995). Tang et al. (1997a,b) reported somatic embryogenesis and plantlet regeneration in slash pine by testing different developmental stages and genotypes of embryos. However, limitations due to low initiation frequency and the genetic specificity of explants are problems associated with somatic embryogenesis when immature zygotic embryos were used as explants. Some of above problems can be resolved by modifying the tissue culture protocol. With new technologies being developed for forestry, slash pine research and technology development has focused on genetics, biotechnology, precision management, intensive management, economics, insect and disease resistance, and genomics. Research work of scientists and practitioners based on somatic embryogenesis and gene transfer will provide new technologies and benefits to foresters and landowners. This chapter describes the protocol to regenerate slash pine plantlets via somatic embryogenesis from embryogenic cultures derived from zygotic embryos.

2. MATERIALS

1. Slash pine female cones at different developmental stages
2. Laminar-flow hood with ultraviolet light, Petri dishes, forceps, scalpel, 1ml to 20 ml serological pipettes, and 10µl to 1000µl air-displacement piston pipettes
3. Mercuric chloride, ethanol, sterile distilled water, 125 ml Erlenmeyer flasks

4. Dissecting microscope
5. 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzyladenine (BA), kinetin (Kn), abscisic acid (ABA), indole-butyric acid (IBA), gibberellic acid (GA_3), tissue culture agar, and sucrose
6. Myo-inositol, casein hydrolysate, and L-glutamine, polyethylene glycol (PEG), and activated charcoal
7. Tissue culture chambers and shaking incubators
8. Media (see Tables 1 and 2)

Table 1: SLASH PINE BASIC CULTURE MEDIUM

Chemicals	**Quantity in mg/l**	**Chemicals**	**Quantity in mg/l**
$Ca(NO_3)_2.4H_2O$	600	$Na_2MoO_4.2H_2O$	0.125
KNO_3	900	$CoCl_2.6H2O$	0.0125
$CaCl_2.2H_2O$	500	$CuSO_4.7H_2O$	0. 0125
NH_4NO_3	1516	$FeSO_4.7H_2O$	15
$MgSO_4.7H_2O$	180	$Na_2EDTA.2H_2O$	20
KI	0.04	Myo-inositol	500
KH_2PO_4	135	Nicotinic acid	0.1
$ZnSO_4.7H_2O$	4.3	Pyridoxin HCl	0.1
$MnSO_4.H_2O$	0.5	Thiamine HCl	0.2
HBO_3	3.1	Glycine	0.4
KI	0.83	pH	5.8

The composition of supplementary organic compounds of the modified LP medium is Lysine 100 mg/l, L-glutamine 200 mg/l, L-alanine 0.05 mg/l, L-cysteine 0.02 mg/l, L-arginine 0.01 mg/l, L-leucine 0.01 mg/l, L-phenglalanine 0.01 mg/l, L-tyrosine 0.01 mg/l, D-xylose 150 mg/l, D-glucose 180 mg/l, D-arabinose 150 mg/l, L-maltose 360 mg/l, L-galactose 180 mg/l, L-fructose 180 mg/l, and L-mannose 150 mg/l (von Arnold and Eriksson 1979). The pH was adjusted to 5.8 with 1N KOH or 0.5 N HCl prior to autoclaving at 121^{o}C for 18 min.

Basic medium composition is listed in Table 1. Required modifications for different culture stages are listed in Table 2. The pH is adjusted to 5.8 with 1N KOH or 0.5 N HCl prior to autoclaving at 121^{o}C for 18 min. Filter 2,4-D, BA, IBA, Kn, ABA, GA_3, to sterilize and add to sterile media aseptically. Pour 40 ml medium to 125 ml Erlenmeyer flasks or 20 ml medium to 15mm ×100mm Petri dishes.

3. METHOD

The regeneration procedure includes five steps: (1) embryogenic culture initiation from explants, (2) maintenance and proliferation of embryogenic cultures, (3) embryo development, (4) maturation, and (5) embryo germination and acclimatization and field transfer.

TABLE 2: FORMULATIONS OF SLASH PINE MEDIA

Chemicals	**LP-1**	**LP-2**	**LP-3**	**LP-4**	**LP-5**
	Stage I Initiation	Stage II Maintenance	Stage III Development	Stage IV Maturation	Stage V Germination
Myo-Inositol	400[(1)]	400	800	-	-
L-Glutamine	400	400	400	-	-
Casein hydrolysate	400	400	400	-	-
Sucrose	30,000	30,000	30,000	15,000	15,000
PEG 6000	-	-	-	75,000	-
2,4-D	8	1	1	-	-
IBA	-	-	-	-	0.5
BA	4	0.5	0.5	0.5	-
Kinetin	-	-	0.4	-	-
ABA	-	-	-	4	-
GA_3	-	-	-	-	0.1
Activated charcoal	-	-	-	5,000	500
Agar[(2)]	7,000	6,500	6,500	6,500	6,500

[(1)]All units are in mg/l
[(2)]Tissue culture agar, not used for liquid media
The pH of all media are adjusted to 5.8

3.1. Embryogenic Culture Initiation

Use immature zygotic embryos for embryogenic culture initiation. Collect female cones in early July to late August. Store cones with seeds in plastic bags at 4°C for 2 months before use.

1. Remove the seeds from the immature cones.
2. Rinse the seeds with tap water and agitate for 30 min.
3. Treat the seeds with 70% v/v ethanol for 45 sec.
4. Wash the seeds 5 times with sterile distilled water, 2 min each time.
5. Sterilize the seeds with 0.1% (w/v) mercuric chloride and shake for 20 min.
6. Rinse the seeds 5 times with sterile distilled water in the laminar-flow hood, 2 min each time.
7. Transfer the sterile seeds into a Petri dish.
8. Remove the seed coat with sterile scalpel and forceps and aseptically isolate immature zygotic embryos from the megagametophyte under a dissecting microscope.

9. Place the isolated zygotic embryos horizontally on the surface of 20 ml of gelled callus induction medium in 15mm × 100mm Petri dishes plates or 40 ml of gelled callus induction medium in 125 ml Erlenmeyer flasks. Make sure the whole embryos are touching the medium.
10. Incubate the embryos in darkness at 23°C.

3.2. Maintenance and Proliferation of Embryogenic Cultures

1. Callus is formed on radicles of immature zygotic embryos after 9 weeks on callus induction medium (LP-1). White to translucent, glossy, mucilaginous callus develops from the hypocotyl as well as from the radicle of the immature zygotic embryos explants.
2. White to translucent, glossy, mucilaginous callus is embryogenic (Fig. 1A) and is proliferated on solidified LP-2 medium with 1mg/l 2,4-D, 0.5 mg/l BA, 400 mg/l myo-inositol, 400 mg/l casein hydrolysate (CH), 400 mg/l L-glutamine, and 3% sucrose.

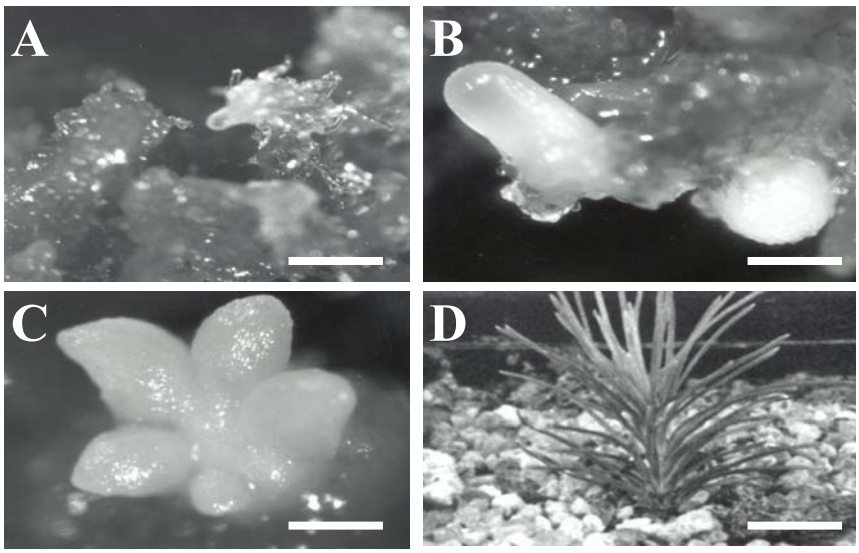

Figure 1 Plant regeneration via somatic embryogenesis in slash pine.
(A) Embryogenic callus obtained from zygotic embryo (bar = 0.1 cm); (B) Globular somatic embryo (bar = 0.5 cm); (C) Cotyledonary somatic embryos (bar = 0.5 cm); (D) Regenerated plantlet established in soil (bar = 0.8 cm)

3. After an additional 9 weeks on the LP-2 medium, white to translucent, glossy, mucilaginous calli containing embryogenic suspensor masses (ESMs) is observed. It is subsequently transferred to a medium consisting of LP basic medium as described above, but supplemented with 1 mg/l 2,4-D, 0.5 mg/l BA, 0.4 mg/l Kn, 800mg/l myo-inositol, 400mg/l casein hydrolysate (CH), 400 mg/l L-glutamine, and 3 % sucrose (LP-3).
4. The ESMs cultures are maintained by subculture every three weeks on fresh LP-3 medium and incubated in darkness at 23^{o}C.

3.3. Making of Cell Suspension Cultures

1. Transfer 1 g of embryogenic cultures to a liquid proliferation medium supplemented with 1 mg/l 2,4-D, 0.5 mg/l BA, 400 mg/l myo-inositol, 400 mg/l CH, and 400 mg/l L-glutamine, and 3% sucrose.
2. Place the flask on a rotary shaker with shaking at 150 rpm in the light with a photoperiod of 16 h (40 μmol $m^{-2}s^{-1}$ cool white fluorescent lamps). The resulting liquid suspension cultures consist of small cell clusters, ESMs, and single cells.
3. During the early stages, cultures are filtered through a 500 μm stainless steel sieve every two days, then cultures are collected on a 100 μm metal sieve (centrifuged at 3000 rpm for 5 min) and re-suspended in fresh medium at a density of 1ml packed cell volume (PCV means the measurement of the ratio of the volume occupied by the cells to the volume of the whole suspension cultures in a sample of cell suspension cultures; this ratio is measured after appropriate centrifugation and is expressed as a decimal fraction) per 50 ml.
4. The suspension cultures are sub-cultured weekly. After tthree weeks, embryogenic cells and ESMs are established. ESMs are defined as polarized structures organized into an embryogenic region subtended by elongated suspensor cells (Attree and Fowke 1993). Proliferating embryogenic tissues consist primarily of single embryogenic cells, ESMs and immature somatic embryos. ESMs continuously initiate embryos by cleavage polyembryogenesis.
5. Due to the organized nature of conifer tissues in solidified media, embryogenic tissue needs to be transferred to liquid media. Liquid cultures are capable of continued embryo proliferation and can remain embryogenic following prolonged culture for more than one year (Attree et al. 1990). This attribute is very useful in large-scale production of somatic embryos and artificial seeds (Attree and Fowke, 1993; Becwar and Pullman, 1995; Gupta et al. 1993; Tautorus et al. 1991).

3.4. Staining to confirm embryogenic nature

The double staining method described by Gupta and Durzan (1987) is used to confirm the presence of ESMs in embryogenic calli and cell suspension cultures.

1. Small pieces of embryogenic cultures with ESM are submerged in a few drops of 1% (w/v) acetocarmine and heated for 5 sec.
2. Wash embryogenic cultures with liquid medium and stain with 0.05% Evan's blue for 10 sec.
3. The embryonal head cells stain bright red and the suspensor cells stained blue. Double staining of embryonal suspensor masses reveal the presence of numerous early stage somatic embryos with suspensor cells in the white to translucent, glossy, mucilagious callus.
4. Embryonal suspensor masses consist of the embryonal head and elongated suspensor cells. The embryonal head consists of the smaller cells with large nuclei and dense cytoplasm.

3.5. Embryo Development, Maturation, and Germination

To obtain the maturation of slash pine somatic embryos, embryogenic cultures containing ESMs need to be transferred from an environment that promotes cleavage polyembryogenesis to one containing abscisic acid (ABA), polyethylene alcohol (PEG), and activated charcoal. ABA prevents the developing embryos from germinating precociously. PEG stimulates the maturation of somatic embryos by regulating their osmotic potential. Activated charcoal absorbs harmful compounds and hormones in tissues and encourages the maturation of somatic embryos (Attree and Fowke, 1993; Gupta et al. 1993; Tautorus et al. 1991).

1. Transfer ESMs suspension cultures with stage 1 embryos (small embryos consisting of an embryogenic region of small, densely cytoplasmic cells subtended by a suspensor comprised of long and highly vacuolated cells) on a proliferation medium with auxin and cytokinin (LP-2).
2. Transfer ESMs suspension cultures with stage 2 embryos (embryos with a prominent embryogenic region that is more opaque and with a more smooth and glossy surface than stage 1 embryos) (Fig. 1B) on a proliferation medium with decreased auxin and cytokinin concentrations (LP-3).
3. Transfer ESMs suspension cultures with stage 3 embryos (embryos with small cotyledons) to medium devoid of auxin (LP-4).
4. Transfer ESMs suspension cultures with stage 4 embryos (embryos with fully developed cotyledons) on LP-5.

5. Mature somatic embryos with cotyledons (Fig. 1C) are transferred to solidified LP medium containing 0.5 mg/l IBA, 0.1 mg/l gibberellic acid (GA_3), and 0.05% activated charcoal for 4-12 weeks.
6. Somatic embryo desiccation is performed according to the method of Tang (2000). Embryos in a petri dish are dried through a series of desiccators in which the relative humidity (RH) is kept constant using a saturated solution of K_2SO_4 (RH 87%), Na_2CO_3 (RH 80%), NaCl (RH 70%), NH_4NO_3 (RH 61%), or $Ca(NO_3)_2 \cdot 4H_2O$ (RH 50%) (Tang 2000). They are transferred daily from a desiccator at a higher RH to one at a lower RH.
7. When somatic embryos begin to grow epicotyls and priminary roots, all the germinated plantlets are transferred to gelled LP-5 medium containing 0.5 mg/l IBA, 0.1 mg/l gibberellic acid (GA_3), and 0.05% activated charcoal for further development.

3.6. Acclimatization and Field Transfer

1. The morphologically normal plantlets with both shoots and roots that develop from somatic embryos are transferred to square plastic pots (Fisher Scientific) containing a perlite: peatmoss: vermiculite (1:1:1 v/v) mixture located in a greenhouse (Fig. 1D).
2. For acclimatization, plantlets are covered with glass beakers for one week. Then the acclimatized plantlets are exposed to greenhouse conditions by removing the cover for 16 days.
3. Then the plants are transplanted to soil in the field. Eight weeks after planting, the survival rate of regenerated plants can be determined.
4. Following in vitro culture, regenerated plantlets require a gradual decrease in relative humidity to acclimatize to greenhouse conditions prior to planting in the field. After partial drying (desiccation) and acclimatization, somatic embryos develop into regenerated plantlets with functional apical meristems.

Without partial drying and acclimatization, somatic embryos often fail to form functional shoot meristems during subsequent growth, and the resulting plantlets have a lower survival rate. Regenerated plantlets more than 3 cm in height are transferred to an autoclaved perlite: peatmoss: vermiculite mixture (1:1:1 v/v). Their survival rate is dependent upon the acclimatization time. The highest survival rate of regenerated plantlets is obtained when the acclimatization time was 16 days.

4. IDENTIFY STEPS REQUIRED FURTHER PROTOCOL MODIFICATIONS

Plant regeneration via somatic embryogenesis in slash pine is described in this protocol. However, further protocol modifications are needed. These include: (1) improved somatic embryo development and maturation; (2) increased frequency of somatic embryo germination; (3) establishing an effective genetic transformation system using embryogenic cultures; (4) improving the method of artificial seed production, and (5) developing bioreactors that can be used for large-scale production of somatic embryos (Handley, 1998; Hakman and Fowke 1987).

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SOMATIC EMBRYOGENESIS AND GENETIC TRANSFORMATION IN *PINUS RADIATA*

Christian Walter, Jens I. Find and Lynette J. Grace

New Zealand Forest Research Institute Ltd, Sala Street, Rotorua, New Zealand
Email corresponding author: christian.walter@forestresearch.co.nz

1. INTRODUCTION

Pinus radiata (radiata pine) is a coniferous gymnosperm native to California. It is also called Monterey pine, indicating its native habitat where it was first described. Radiata pine has been widely used as a plantation tree species in other countries, and the bulk of plantations (a total of around 4 million hectare) are mainly found in the Southern Hemisphere, in New Zealand, Chile and Australia (Menzies and Aimers-Halliday, in press). The climate in these countries allows fast growth rates and rotation times around 25 to 30 years are common. The species is used as a plantation tree (clonal or family forestry) and is making significant contributions to the export earnings. Intensive selection and breeding programs, particularly in New Zealand, have led to the production of superior trees for clonal or family forest plantations, which show significantly increased growth rate, better form and resistance to specific pests or environmental adverse conditions. Clonal propagation technologies available include embryogenesis and various cuttings technologies, all of which are now used on commercial scales (Menzies and Aimers-Halliday, in press; White and Carson, in press).

However, many desirable attributes are not available in the breeding population nor in the original genetic resource and new biotechnologies such as genetic engineering have been developed to introduce specific genes from other organisms (Bishop-Hurley 2000; Walter et al, 1998).

This chapter focuses on a protocol for radiata pine embryogenesis and regeneration, which are prerequisite for the successful genetic transformation of many conifer species. A Biolistic transformation protocol will then be described in detail, along with protocols for molecular analysis of transgenic tissue and plant material. The protocol has been optimised in our lab for the genetic modification of radiata pine (Walter et al, 1998), however we have shown successful

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transformation of *Picea abies*, *Pinus taeda* and *Abies nordmanniana* as well, using this method (Walter et al, 1999; LJ Grace, pers comm.; JI Find, in prep.).

2. INDUCTION OF SOMATIC EMBRYOGENESIS AND PLANT REGENERATION

2.1. Initiation

Radiata pine embryogenic tissue is initiated by culturing whole megagametophytes containing immature embryos at bullet-to pre-cotyledonary stage. The optimum time for cone collection is from the first to the third week of January (approximately 8–10 weeks after fertilisation) depending on the seed family sampled. Cones are usually processed within 48 hours but we have stored them in the dark at 4°C for a month with no detrimental effect on initiation or contamination. Seeds are removed from the cones, surface sterilised in 10 % H_2O_2 (30% reagent grade) containing 1 drop of Silwet (Union Chemicals, Auckland, NZ) for 10 minutes, then rinsed 2-3 times in sterile water. The seed coat is removed aseptically and the megagametophyte is placed onto initiation medium (EDM6: Table 1) (Smith 1996). We have also found that initiation can be achieved, and is sometimes improved, by culturing embryos dissected from the seed. Six megagametophytes or 7 embryos are cultured per Petri dish (90x25mm) and are incubated at 24 +/- 1°C in dim light (5µmols m^{-2} s^{-1}). After 2 to 6 weeks the embryos from within the megagametophyte are expelled onto the medium and embryogenic tissue develops due to cleavage polyembryony. Once the embryogenic tissue mass reaches 10mm in diameter, it is separated from the original explant and transferred to maintenance medium.

2.2. Maintenance

Embryogenic tissue is maintained by serial subculture to fresh medium every 14 days. Maintenance conditions are the same as for initiation. Embryogenic cultures can alternatively be maintained on BLG1 (Table 1). In general, cultures that are maintained on BLG1 remain in a less differentiated state and maintain their maturation capacity for longer than those on EDM6.

Suspension cultures are established by transferring 2-3g of embryogenic tissue from solid medium into 25 ml liquid BLG1 medium in baffled 250 ml Erlenmeyer flasks. The flasks are sealed and placed on a rotary shaker at 70 rpm in the dark at 24°C. During the establishment phase, BLG1 medium is added weekly on the basis of growth until a volume of approximately 100 ml is reached, usually after 3-4 weeks. When the liquid cultures are established, they are maintained by weekly subculture with fresh BLG 1 medium. The suspension culture is transferred to a 100 ml measuring flask and allowed to settle for 30min. Surplus liquid and tissue

is discarded and fresh medium is added to the settled cell volume (SCV) at a ratio of 4:1 (v/v).

Table 1: Formulations of basal culture media

Component	***EDM6***	***BLG1***	***EMM1/EMM2***	***BLG6***	***BMG-2***
	Concentration mg/1				
Inorganic Salts					
KNO_3	1431	100	1431	100	506
$MgSO_4*7H_20$	400	320	400	320	493
$CaC1_2*2h_20$	25	440	25	440	-
$NaNO_3$	310	-	310	-	-
$NH_4H_2P0_4$	225	-	225	-	-
KC1	-	-	-	-	149
NH_4N0_3	-	-	-	-	320
KH_2P0_4	-	-	-	-	272
$Ca(N0_3)_2*4H_20$	-	-	-	-	709
$MnS0_4*4H_20$	3.6	16.9	3.6	16.9	4.22
H_3B0_3	8	6.2	8	6.2	4.65
$ZnS0_4*7H_20$	25	8.6	25	8.6	1.44
KI	1	0.83	1	0.83	0.083
$CuS0_4*5H_20$	2.4	0.025	2.4	0.025	0.25
$Na_2Mo0_4*2H_20$	0.2	0.25	0.2	0.25	0.121
$CoCI_2*6H_20$	0.2	0.025	0.2	0.025	0.012
$FESO_4*7H_20$	30	27.8	30	27.8	-
Na_2EDTA	40	37.3	40	37.3	-
NaFe EDTA	-	-	-	-	32.5
$NiCI_2$	-	-	-	-	0.024
$A1C1_3$	-	-	-	-	0.024
Vitamins					
Thiamine.HCL	5	1	5	1	1
Nicotinic acid	5	0.5	5	0.5	0.5
Pyridoxine.HCL	0.5	0.5	0.5	0.5	0.5
Amino Acids					
Glutamine	550	1450	7300	1450	-
Asparagine	525	1000	2100	1000	-
Arginine	175	-	700	-	-
L-Citrulline	19.75	-	79	-	-

L-Ornithine	19	-	76	-	-
L-Lysine	13.75	-	55	-	-
L-Alanine	10	-	40	-	-
L-Proline	8.75	-	35	-	-
Glycine	-	2	-	2	2
Myo-inositol	1000	100	1000	100	100
Sucrose	30000	30000	30000	30000	20000
Gelrite	3000	-	EMM1: 6000 EMM2:4500	-	-
Phytagel	-	1800	-	1800	3500
Charcoal	-	-	-	-	10000
Growth regulators					
2,4-D	1	1	-	-	-
BA	0.6	0.6	-	-	-
ABA	-	-	15	15	-

pH of all med adjusted to 5.7
Amino acids and ABA are filter sterilised and added to cooled medium

2.3. Maturation

Radiata pine somatic embryos can be produced using either of two protocols depending on the medium the embryogenic tissue is maintained on.

Protocol 1: Five portions (10 mm diameter) of embryogenic tissue, taken 7 days after transfer to EDM6, are placed on Embryo Maturation Medium (EMM1: Table 1) (Smith 1996) and cultured for 14 days. Tissue pieces are then divided and portions are placed on a second maturation medium (EMM2: Table 1), which has the same composition as EMM1 except for a lower gelrite concentration (0.45% v 0.6%). Tissue is divided and transferred to fresh EMM2 every 14 days until mature embryos appear (6-8 weeks).

Protocol 2: Two weeks after subculture on BLG 1 (7 days for suspension cultures) embryogenic tissue is suspended as 1g tissue in 5ml BLG 1 without growth regulators. Filter papers (Whatmann #1) are placed on BLG 6 medium (Table 1) and aliquots of 1ml of embryogenic culture are spread on each filter paper with a wide mouth pipette tip. Filters with tissue are transferred to fresh medium every 2 weeks until mature embryos have developed.

For both protocols, tissue is incubated at 24 +/- 1°C in dim light (5µmols m^{-2} s^{-1}).

2.4. Somatic Embryo Germination and Transfer to Soil

Somatic embryos which are white, waxy with well formed cotyledons (Fig.1A) are harvested from tissue and transferred onto nylon cloth (30μm) contained in each of 3 wells of a 6 well Falcon Multiwell® dish. The remaining 3 wells are half filled with sterile water. The dish perimeter is sealed with plastic food wrap and the entire dish is wrapped in tinfoil and stored at 5°C for at least 7 days. This treatment has been found to synchronise and speed root emergence. The nylon cloth containing the embryos is transferred to germination medium (BMG-2: Table 1) (Krogstrup 1991) and incubated for 7 days at 24/20°C under lights (90 μmol m^{-2} s^{-1}) with a 16 h photoperiod. Embryos are then removed from the nylon cloth and placed horizontally on the medium. When the somatic seedlings have germinated and developed epicotyls (6-8 weeks, Fig 1B) they are transplanted into Hyco V50 trays containing a mixture of peat: pumice (2:1 ratio). The tray is covered with a plastic lid that is removed for increasing periods of time over a 2-week period to gradually acclimatise the seedlings to greenhouse conditions. Seedlings are hand misted twice a day for the first 5-7 days then once a day from then on. The greenhouse is operated with a day/night temperature of 18/12°C and a 16h photoperiod (Fig 1D).

Embryogenic tissue can be cryopreserved in liquid nitrogen. It can be stored for many years, potentially for decades. (For protocols refer to Hargreaves and Smith, 1994 a&b)

3. GENETIC TRANSFORMATION OF EMBRYOGENIC RADIATA PINE TISSUE

Genetic engineering in conifers usually depends on the availability of both tissue culture techniques allowing regeneration of a plant from a few (embryogenic) cells and on DNA transfer methods enabling the introduction and establishment of foreign genes into the genome of such cells (Walter et al. 1998 and 2002; Bishop-Hurley et al, 2000). Somatic embryogenesis (SE) has been used frequently to achieve this goal and the advantages of SE for transformation include: 1) tissue can be proliferated rapidly, either in liquid or on solid medium; 2) tissue can be temporarily suspended in liquid and plated on medium in a thin layer which allows for easier selection of resistant cells following genetic transformation; 3) plants can often be regenerated from single cells potentially resulting in lower frequencies of chimera formation; 4) individual transformed lines (transclones) can be cryopreserved while plants are regenerated and tested in containment or in the field, and 5) embryogenic suspensions provide a source of rapidly dividing cells which has been shown advantageous for transformation (Iida et al.,1991; Sangwan et al., 1992).

Genetic engineering can be used to create lines that display desirable new characteristics, or to modify existing ones in a way that has not been possible previously. New traits are usually introduced through over-expression of heterologous genes, whereas existing traits can be modified by suppression (antisense or RNAi approaches) of endogenous genes (Bishop Hurley et al., 2000; Tang and Tian, 2003; Li et al, 2003; Waterhouse and Helliwell, 2003).

Genetically engineered conifers became a reality in the early 1990s (Huang et al., 1991) and since then many protocols have been published for a range of conifers (Charest et al., 1993; Ellis et al., 1993; Klimaszewska et al., 2001; Tang and Tian, 2003; Walter et al, 1998 and 1999). Both Biolistic and *Agrobacterium* mediated transformation have been reported, however at least in the early years, the development of *Agrobacterium* transformation has been difficult due to the recalcitrance of *Agrobacterium* to infect conifers (or the ability of conifers to successfully defend against pathogen attack). Here we introduce Biolistic transformation technologies in more detail. Details on *Agrobacterium* transformation of conifers can be found in (Huang et al 1991; Klimaszewska et al, 2001; Charity et al, in press).

3.1. Biolistic Transformation

Artificial genetic engineering protocols for plants involve various approaches to introduce genes in the form of pure DNA into plant cells. While electroporation of cells has been reported for a range of species (for example maize: D'Halluin et al., 1992; rice: Battraw and Hall, 1992), no routine genetic engineering protocol using electroporation has been published for conifers. Another direct DNA transfer method is Biolistics , whereby small (1-3µm diameter) spherical bullets are coated with DNA and shot into target cells. These bullets are usually made of gold or tungsten, and DNA may integrate into the genome of the cells if they are in a competent physiological state and the physical conditions for delivery are appropriate for the species under investigation (Klein et al., 1987). Biolistic techniques have successfully been applied to conifer transformation followed by the regeneration of transgenic plants. A standard Biolistic transformation protocol routinely used for *Pinus radiata* (Walter et al, 1998) includes embryogenic tissue, and is described in detail.

3.2. Preparations for Biolistic transformation:

One day before bombardment, embryogenic tissue is subcultured from a liquid suspension (1g tissue suspended in 5ml BLG 1 medium, without growth regulators. 1ml of this suspension is spread onto a Whatman filter disc on solid BLG 1 medium and the Petri dish with lid is left in the laminar flow overnight for excess

water to evaporate. This is an important step because any film of water remaining on top of embryogenic cells will stop gold particles from penetrating the cell walls. Gold particles (1.5 to 3.0 micron, Aldrich Chemicals, MI, USA) are coated with plasmid DNA using a published procedure (Sanford et al, 1993; Walter et al, 1994).

For bombardment, the following physical conditions should be used (optimised for DuPont PDS1000 He): Gap distance: 6mm; macrocarrier travel distance: 16mm; microcarrier travel distance: 6cm; rupture disc pressure: 1350psi; vacuum in chamber: 25in Hg.

3.3. Selection of transgenic tissue:

By introducing an antibiotic selection gene (for example *npt*II or *aph*IV), selection of transgenic lines (transclones) should be carried out (Wagner et al.1997)) to differentiate transformed cells from non-transformed cells. Other selection genes, for example the *bar* gene for resistance against phosphinothricin, does not appear useful in *P. radiata* transformation, but has been used as a selective agent for *Picea abies* embryogenic tissue (Clapham et al., 2000). When designing a selection protocol for a given species and a specific line (genotype) of embryogenic tissue, the required level of selective pressure should be determined very carefully. When using geneticin as selective agent for example, a range of 5-35mg/l should be tested with non-transgenic tissue. The smallest concentration that completely inhibits growth of embryogenic cells on selection medium should be used for selection of transgenic lines. Also, a non-bombarded control should always be included in a transformation experiment to make sure that the chosen selection level is still appropriate to kill non-transgenic tissue. In our laboratory, we have frequently found that embryogenic tissue of the same genotype can display different levels of resistance to geneticin, at different times. Visual selective markers such as GFP and non-antibiotic selection strategies may also be useful for conifer transformation, however no results have been published yet.

Following transformation using a Biolistic device, embryogenic cells on filters are transferred to selection medium where they remain for up to 3 months. Some researchers prefer to subculture the cells on fresh selection medium every 2-3 weeks. In radiata pine, selection is usually very stringent and escapes are hardly ever seen

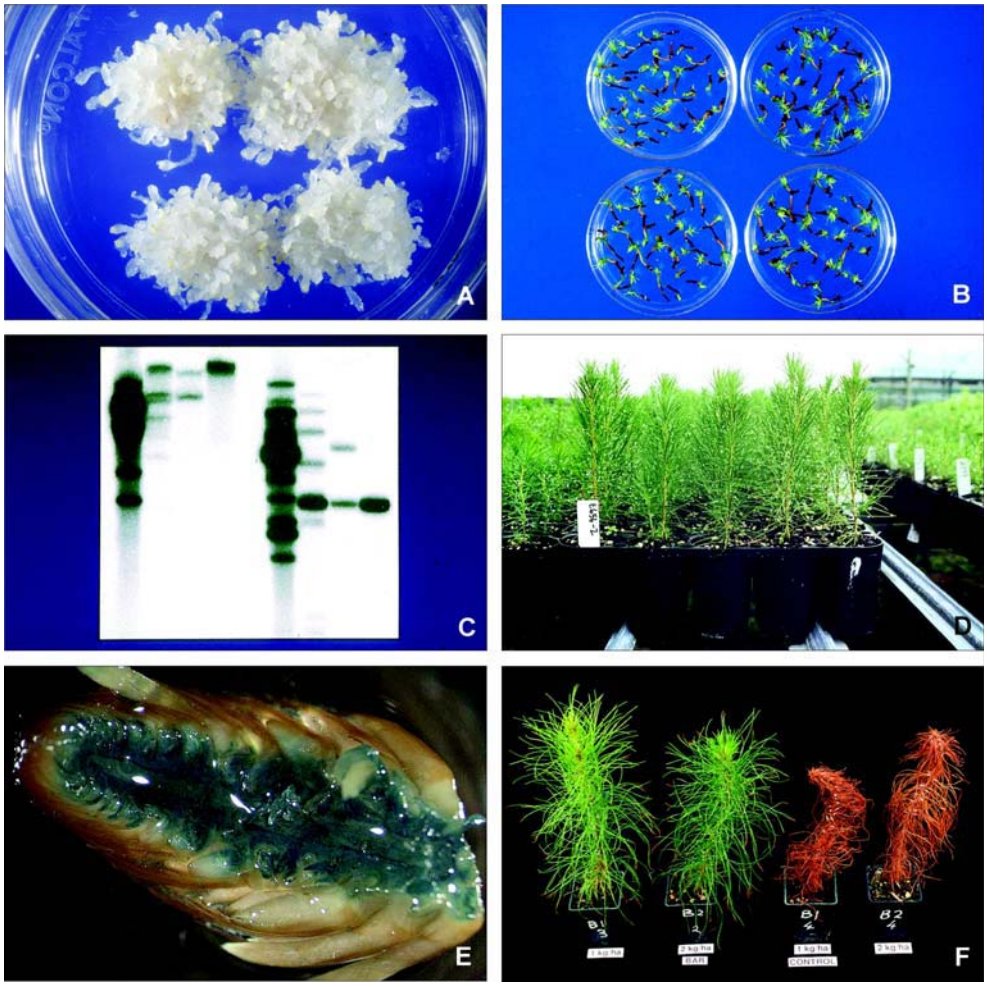

Figure 1: *Pinus radiata* embryogenesis and transformation. A: Embryogenic tissue grown on Maturation Medium; B: Embryos regenerating and germinating. C: Southern hybridisation analysis of transgenic *P radiata* trees; D: Trees grown from somatic seedlings of *P radiata*; E: Cross section through a vegetative bud of an adult plant transformed with the *uid*A gene and stained for *uid*A expression; F: Herbicide resistance in radiata pine. Transgenic plantlets with a gene conferring resistance against the herbicicde phosphinothricin and survive spraying with the herbicide (left) control plantlets (right) dead (Bishop-Hurley et al, 2001).

Vigorously growing pieces of tissue on selective media are good candidates for further molecular analysis for stable integration of new genes. In the following, we describe a range of routine analysis techniques used in our laboratory:

3.4. Southern hybridisation to confirm successful transformation and to evaluate complexity of transgene integration:

Southern hybridisation analysis of transgenic plants derived from a Biolistic® transformation experiment should be used to confirm the presence of transgenes, and to determine their copy number (Fig. 1c). This is particularly important since the use of Biolistic transformation technologies can result in very high copy numbers of the introduced gene and also in fragmented copies integrated into the genome. In some cases, several hundred copies of the transgene appear to be present in the transgenic tissue (Walter et al, 1998; Fig.1c). High copy numbers and fragmented copies may have negative effects on gene expression stability and correct long-term gene expression, however it is still not demonstrated that this holds true for conifers. The issue of gene silencing and gene arrangements based on multiple copies and fragmented copies, appears of great importance to genetic engineering in trees, since these organisms will be around many years and are expected to express transgenes correctly, over a period of 30 years or more. Field trials with transgenic conifers will help to better understand this phenomenon in trees including conifers, and possibly lead to strategies to avoid silencing and expression-instability (Kumar and Fladung, 2001; Fladung 1999).

Therefore, it may be prudent to carry out Southern hybridisation analysis with all transgenic lines and use only those for plant regeneration that show relatively low complexities of integration. However, results from our experiments with three to six year old transgenic *Picea abies and P. radiata*, indicate a high stability of transgene expression over this period of time and mainly independent of copy numbers. It is possible that conifers, having a relatively large genome as compared to other plant species, show lower levels of gene silencing than other species, or perhaps none at all.

Many different protocols have been published on DNA isolation from plant tissue and there are some efficient and economic procedures offered by commercial manufacturers. However we prefer to use a method for genomic DNA isolation modified after Doyle (1990). The protocol starts with 1-2 g of fresh tissue (needles or embryogenic tissue), which is grounded in liquid nitrogen with 200 mg acid-washed sand (SIGMA). The powder is then transferred to 15 ml extraction buffer (2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris HCl pH 8.0, 1% w/v polyvinylpyrollidone and 0.2% w/v ß-mercaptoethanol; (SIGMA) preheated at 65°C. Samples are incubated at 65°C for about 30 min with occasional swirling,

then mixed twice with an equal volume of chloroform-isoamyl-alcohol (samples are centrifuged at 1,600g for 5min and the supernatant transferred to a fresh tube). An equal volume of ice-cold isopropanol is poured onto the aqueous phase and genomic DNA is removed from the sample after 5min at room temperature by using a glass hook, or by centrifugation at 14,000 rpm for 5min in a Biofuge 13 benchtop centrifuge (Heraeus, Osterode, Germany). Genomic DNA should then be resuspended in 2.5ml Tris-EDTA with RNAase A (10 µg/ml, Boehringer, Mannheim, Germany) at 37°C for 15min, and subsequently incubated at room temperature for 30 min. An equal volume of 4 M NaCl is added to the sample and incubated at 37°C for 30min, then allowed to cool. Insoluble material is removed by centrifugation (5min at 10,000 rpm in a Sorvall RC5C centrifuge, SS34 rotor) (Sorvall/DuPont, Newton, CA, USA) and the supernatant is transferred to a second tube avoiding any interphase material. An equal volume of isopropanol is poured onto the supernatant and the genomic DNA removed with a glass hook, washed with 70% v/v ethanol, air dried and resuspended in 1 ml TE (pH 8.0). The quality of DNA can be checked by gel-electrophoresis (1% w/v gel, 100 V for 1 hour).

For Southern hybridisation, a minimum of 30µg genomic DNA should be used per lane. The DNA is precipitated with ethanol, and then resuspended in loading buffer (SUDS, Sambrook et al, 1989) and run on a 1% w/v agarose gel (100V, 2h). The nucleic acids are hydrolysed with 0.25 M HCl and subsequently transferred to Hybond N+ membranes (Amersham, Little Chalfort, England) using alkali transfer buffer (0.4 M NaOH). After transfer (3h), membranes are rinsed in 5 x SSC, transferred to hybridisation solution (10% dextran sulphate MW~500,000, (Pharmacia, Uppsala, Sweden), 1% w/v SDS, 6% w/v NaCl, with 2 x Denhardts reagent and incubated at 65°C overnight. Probes (amplified by PCR, primers as described in PCR analysis) should be random labelled with 32P (Amersham Ready Prime system) and separated from unincorporated nucleotides as recommended by the manufacturer. Hybridisation is carried out according to standard protocols (Sambrook et al., 1989). Blots are washed (2.5 min in 2 x SSC at room temperature, 2 x 30 min washes in 2 x SSC, 1% w/v SDS at 75°C, 30 min in 0.1 x SSC at room temperature, Sambrook et al, 1989) and exposed to Kodak X-Omat AR film for 3 days, at -80°C. Autoradiograms can be scanned, for example using a digital scanner and processed using image analysis software (for example NIH image software, http://rsb.info.nih.gov/nih-image/).

3.5. Detection of novel proteins in genetically engineered conifer tissue using ELISA techniques for *npt*II and *cry*1Ac

Transgenic conifer tissue can be assayed using ELISA (Enzyme Linked ImmunoSorbent Assay) to confirm the expression of the selective marker *npt*II and the presence of the neomycin-phosphotransferase enzyme in transgenic tissue and plants. The assay is very specific and sensitive and background expression usually